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As a model of epileptogenesis and temporal lobe epilepsy (TLE), status epilepticus (SE) triggers structural and functional neuronal changes that take place during a latent period of weeks or months after SE, followed by the development of spontaneous seizures. Neuronal loss and synaptic reorganizations of limbic structures during epileptogenesis in the rat brain are similar to neuropathological observations in TLE patients. The development of regimens that can inhibit epileptogenesis requires understanding the alterations that occur during epileptogenesis in brain regions that are important in epilepsy.

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ABSTRACT

Pathophysiological alterations in the basolateral amygdala and neurodegeneration of limbic structures during epileptogenesis induced by status epilepticus

by

Felicia Qashu

As a model of epileptogenesis and temporal lobe epilepsy (TLE), status epilepticus (SE) triggers structural and functional neuronal changes that take place during a latent period of weeks or months after SE, followed by the development of spontaneous seizures. Neuronal loss and synaptic reorganizations of limbic structures during epileptogenesis in the rat brain are similar to neuropathological observations in TLE patients. The development of regimens that can inhibit epileptogenesis requires understanding the alterations that occur during epileptogenesis in brain regions that are important in epilepsy. The amygdala plays a central role in the symptomatology and pathogenesis of TLE, and of the amygdala nuclei, the basolateral nucleus of the amygdala (BLA) is the most important for the initiation and spread of seizure activity. Therefore, we investigated alterations in the BLA that accompany epileptogenesis, the extent of neurodegeneration during epileptogenesis in limbic structures important in TLE, and whether termination of prolonged SE with diazepam reduces this neurodegeneration.

In brain sections from adult rats, at 7 to 10 days after SE induced by kainic acid, whole-cell recordings from BLA pyramidal neurons showed a significant reduction in the frequency and amplitude of action potential-dependent spontaneous inhibitory post-synaptic currents (IPSCs), and a reduced frequency but not amplitude of miniature IPSCs

compared to sham rats. The reduced inhibition could be accounted for by the loss of GABAergic neurons, which, with the use of design-based stereology combined with GAD67 immunohistochemistry, was found to be significantly more extensive than the loss of principal cells. A reduced level of the GluR5 kainate receptor subunit, as revealed by Western blot analysis, was accompanied by impairment in the GluR5 kainate receptor-mediated modulation of IPSCs, which could have contributed to the reduction of spontaneous IPSCs. Compensatory mechanisms appeared to be underway, as Western blot analysis revealed an increased level of glutamate decarboxylase and the alpha1 subunit of the $GABA_A$ receptor. Thus, due to a high vulnerability of BLA GABAergic neurons to seizure-induced damage, tonic inhibition in the BLA is significantly reduced at a relatively early stage of epileptogenesis, which may facilitate the progression towards the development of epilepsy.

To compare the extent of neurodegeneration during epileptogenesis in limbic structures, degenerating cells were quantified in the amygdala, hippocampus, piriform cortex, and endopiriform nucleus in brain sections stained with Fluoro-Jade C at 7-10 days after uninterrupted SE, and after SE terminated with diazepam. We found that neurodegeneration at this time point in epileptogenesis is the most extensive in the endopiriform nucleus, followed by the amygdala, with both structures showing significantly more neurodegeneration than the piriform cortex and the hippocampus. Compared to when SE was not interrupted, neurodegeneration was significantly attenuated when SE was terminated after 3 hours with diazepam, in all structures but the hippocampus, with the most protection occurring in the amygdala and piriform cortex. These data suggest that the amygdala, a structure highly important in the development of

TLE and co-morbid mood disorders, undergoes substantial neurodegeneration during epileptogenesis, but is responsive to the $GABA_A$-mediated protection from neuronal loss after prolonged SE.

PATHOPHYSIOLOGICAL ALTERATIONS IN THE BASOLATERAL AMYGDALA AND NEURODEGERATION OF LIMBIC STRUCTURES DURING EPILEPTOGENESIS INDUCED BY STATUS EPILEPTICUS

by

Felicia Qashu

Doctoral Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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LIST OF ABBREVIATIONS

AB – accessory basal nucleus of the amygdala
ACSF – artificial cerebrospinal fluid
AED – anti-epileptic drug
AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATPA – agonist of GluR5 containing kainate receptors
BSA – bovine serum albumin
BZ - benzodiazepine
BLA – basolateral nucleus of the amygdala
CA1 - cornu ammonis field 1
CA3 - cornu ammonis field 3
Ce – central nucleus of the amygdala
CE – coefficient of error
COa – anterior cortical nucleus of the amygdala
CV – cresyl violet
D-APV - specific NMDA receptor antagonist
DAPI - 4',6-diamidino-2-phenylindole, dihydrochloride
DPX - Dibutyl phthalate, Polystyrene granules, xylene mounting medium mixture
DZP – diazepam
EEG - electroencephalograph
En – endopiriform nucleus
FJ – Fluoro-Jade C
GABA - gamma-aminobutyric acid
$GABA_A$ α1 – alpha1 subunit of the gamma-aminobutyric acid receptor type A
GAD – glutamate decarboxylase
GAD65/67 – 65 kD and 67 kD isoforms of glutamate decarboxylase
GAD67 – 67 kD isoform of glutamate decarboxylase
GluR5KR – GluR5-containing kainate receptors
GYKI 52466 – specific AMPA receptor anatagonist
IPI – initial precipitating injury
IPSC - inhibitory post-synaptic current
KA – kainic acid
KA-SE – kainic acid-induced status epilepticus
La – lateral nucleus of the amygdala
Me – medial nucleus of the amygdala
mIPSC – miniature inhibitory post-synaptic current
MFS – mossy fiber sprouting
MTLE – mesial temporal lobe epilepsy
MTS – mesial temporal sclerosis
NGS – normal goat serum
NMDA - N-methyl-D-aspartate
PBS – phosphate buffered saline
Pir – piriform cortex
SCH50911 – specific $GABA_B$ receptor antagonist
SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SE – status epilepticus
SEM – standard error of the mean
sIPSC – spontaneous inhibitory post-synaptic current
TBI – traumatic brain injury
TBS – tris buffered saline
TBS-T - tris buffered saline + Tween-20
TLE – temporal lobe epilepsy
TTX - tetrodotoxin
vEEG - video- electroencephalograph
VPM/VPL - posteromedial and ventral posterolateral thalamic nuclei

CHAPTER 1

Introduction

Temporal Lobe Epilepsy

Epileptic disorders are chronic neurological disorders characterized by the occurrence of spontaneous, recurrent seizures. The etiology, ictal phenomenology, and semiology of epileptic seizures and syndromes are used to categorize epileptic disorders. For example, the terms idiopathic, symptomatic, and cryptogenic are used to describe the etiology of epilepsies of a primary, secondary, or unknown underlying cause, respectively. Idiopathic epilepsies are usually genetically determined, and largely benign, with no identifiable structural lesions (Bendabis, 2001). Symptomatic epilepsies arise secondarily from one or more pathological insults with identifiable lesions, such as head trauma, stroke, tumors, infections, or status epilepticus (Annegers et al., 1980; Salazar et al., 1985; Hesdorffer et al., 1998; Angeleri et al., 1999). Cryptogenic epilepsies have an unidentified underlying cause, and thus are also called presumed symptomatic epilepsies.

Although more diverse ictal phenomena exist, a largely dichotomous classification of epileptic seizures materializes from ictal onset: focal and generalized. In focal seizures (also called “localization-related”, and formerly called “partial”), ictal onset is regional, or limited to one hemisphere, and may become secondarily generalized to both hemispheres. Generalized seizures are characterized by diffuse ictal activity that begins in both hemispheres simultaneously.

Epilepsy disorders are one of the most common neurological disorders, affecting more than 1% of the population (Elger, 2002; Wiestler and Blümcke, 2002). Temporal lobe epilepsy (TLE) is the most common of the focal epilepsies (Engel, 2001; Bendabis, 2001), and is so called TLE because the focal seizures in TLE patients are most often of temporal lobe origin, that can become secondarily generalized (Engel, 1996). The semiology of temporal lobe seizures, based on ictal signs and symptoms, is described by epigastric rising, emotional changes (most commonly fear), and olfactory or gustatory hallucinations (Engel, 1996). Like other symptomatic epileptic syndromes, TLE can be caused by an initial precipitating injury (IPI), such as traumatic brain injury (TBI), vascular insults, cerebral infections, or status epilepticus (Annegers et al., 1980; Salazar et al., 1985; Hesdorffer et al., 1998; Angeleri et al., 1999). In such incidences, the IPI triggers structural and functional neuronal changes that occur during a seizure-free latent period that ranges from weeks to several years (French et al., 1993; Mathern et al., 1995; Treib et al., 1996; Benardo, 2003), culminating with the occurrence of spontaneous seizures; this process, in which the normal brain becomes epileptic, is called epileptogenesis (Figure 1). Current anti-epileptic drugs (AEDs) are used as anticonvulsants to control seizures once epilepsy has developed, however about a third of epilepsy patients are resistant to available AEDs, and an even higher rate of refractoriness is seen in TLE (Leppik, 1992). The latent period between the IPI and appearance of spontaneous seizures offers a time window for anti-epileptogenic therapeutics to prevent or reduce the severity of the developing disorder.

Epilepsy associated with brain insults has been recognized for many years; results from Phase II of the Vietnam Head Injury Study found that 51% of patients with a

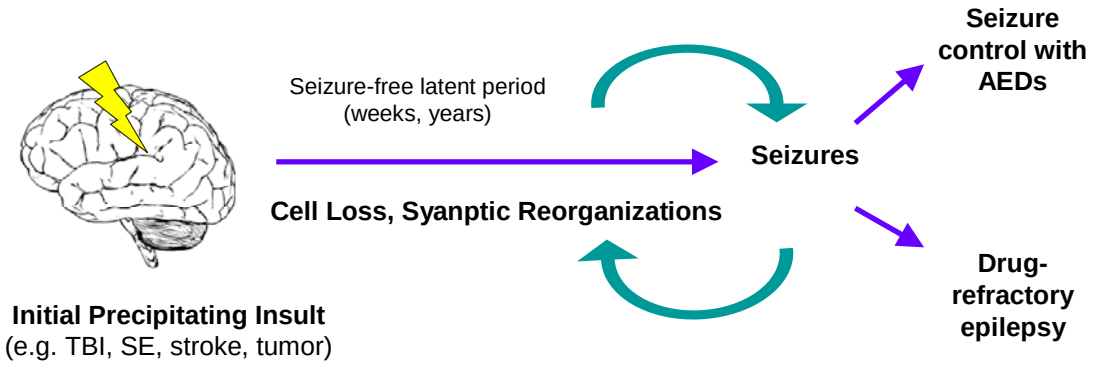

Figure 1: The process of epileptogenesis. An initial precipitating insult such as traumatic brain injury (TBI), stroke, tumor, or an episode of status epilepticus (SE) can trigger epileptogenesis. During a seizure-free latent period of weeks, months, or years, circuit reorganizations and cell death lead to the occurrence of spontaneous seizures and epilepsy. In about 30% of patients, anti-epileptic drugs (AEDs) fail to control seizures; these patients have drug-refractory epilepsy.

penetrating head injury had developed epilepsy 15 years later (Salazar et al., 1985). Although the severity of the TBI is related to the risk of developing epilepsy (Annegers et al., 1998; Skandsen et al., 2008), even mild TBI can trigger epileptogenesis (Annegers et al., 1998). The increasing number of members of the armed services experiencing mild TBI from combat in the Middle East and nearby regions has spawned major interest in the study of pathophysiological, epileptogenic mechanisms.

Several neuropathological features are common among TLE patients; whether these pathologies are a consequence of recurrent seizures, or if they contribute to the epileptogenic process is not well understood. For example, lesions of limbic structures observed in TLE patients can often be attributed to an initial insult, such as a head trauma or cerebral infection (Mathern et al., 1995), but in some patients, there is no history of an underlying initial insult, or no identifiable lesions until after seizures are observed (Stafstrom et al., 1996). Nonetheless, in TLE patients where AEDs fail to control seizures, surgical resection of the hippocampus, amygdala, and/or the parahippocampal cortex often abolishes the seizures (Falconer, 1967), emphasizing the important role of temporal lobe structures in generating and/or maintaining epileptic activity in TLE.

Hippocampal sclerosis is one of the most frequently reported histopathological observations in tissue from TLE patients (Margerison and Corsellis, 1966; Babb and Brown, 1987; Bruton, 1988). In hippocampal sclerosis, pyramidal cells of the CA1 and CA3 regions, granule cells of the dentate gyrus, hilar neurons are preferentially damaged (Babb, 1991), and glial cells are hypertrophied. Because neuronal loss and gliosis commonly extends to other medial temporal lobe structures, such as the amygdala and the entorhinal cortex, the term “mesial temporal sclerosis” (MTS) is often used to describe

the neuropathology of TLE patients (Falconer et al., 1964; Margerison and Corsellis, 1966; Bruton, 1988); in patients which have MTS, and the term mesial temporal lobe epilepsy (MTLE) is therefore sometimes used instead of TLE.

In addition to neuronal loss, aberrant axonal sprouting and synaptic reorganization has also been observed in tissue from TLE patients (Sutula et al., 1989). In the normal hippocampus, dentate granule cells project their mossy fiber axons to CA3 neurons; in the hippocampus of the epileptic human temporal lobe, mossy fiber terminals abnormally found in the supragranular region of the dentate gyrus make synapses on dentate granule cells, forming a recurrent excitatory connection (Sutula et al., 1989). Mossy fiber sprouting (MFS) is detected with Timm immunohistochemistry, which selectively labels mossy fiber terminals due to their high zinc content (Ibata and Otsuka, 1969). Because genetically mutant mice with MFS also have spontaneous seizures (Qiao and Noebels, 1993), and MFS occurs before the development of spontaneous seizures in animal models of TLE (Mello et al., 1993), it is believed that MFS plays an important role in the epileptogenic process.

Additionally, the granule cell layer of the hippocampus is widened in a subpopulation of patients with TLE; this has been termed granule cell dispersion, as granule neurons are found abnormally in the hilus and inner molecular layer of the dentate gyrus (Houser, 1990; Armstrong, 1993). Because seizures induce an increase in neurogenesis in the dentate gyrus in animal models of TLE (Parent et al., 1997; Scharfman et al., 2000), it is hypothesized that granule cell dispersion may result from a malpositioning of newly born granule cells. Increased neurogenesis has not been shown

in the human epileptic hippocampus (Fahrner et al., 2007); granule cell dispersion may conversely be due to aberrant migration of differentiated granule cells.

In addition to cell loss and synaptic reorganizations, glial hypertrophy is also observed in patients with TLE (Eid et al., 2008). Epileptic discharges are in part triggered by an abnormal local depolarization shift that drives neurons into synchronous bursting. These abnormal depolarization shifts can be initiated by extrasynaptic glutamate (Tian et al., 2005). Glial cells, such as astrocytes, can release glutamate, via a calcium-dependent mechanism (Parpura et al., 1994); this observation has prompted the hypothesis that glutamate released by astrocytes is associated with the synchronous firing of neurons that produces epileptic discharges (Heinemann et al., 1999). Thus, the pathological activation of astrocytes may play an important role in epileptogenesis.

TLE Animal Models

A number of animal models are available to study epileptogenesis and TLE; the most common are the status epilepticus induced TLE and kindling models in rodents (Table 1). The kindling model is characterized by daily electrical stimulations of limbic structures *in vivo* that are initially sub-threshold for seizure generation, and, after a number of stimulations, generate electrographic after-discharges and seizures of increasing intensity and duration (Goddard, 1967). Kindling has been associated with TLE because kindled seizures are focal with limbic origin, that can become secondarily

Table 1: Features of animal models of epileptogenesis that are similar to observations in patients with temporal lobe epilepsy (TLE)

	Latent Period	Spontaneous Seizures	Pathology Similar to TLE	References
Kindling	No	No	•Synaptic reorganizations •Cell loss •Memory Impairments	• Sutula et al., 1988 • Kotloski et al., 2002; Löscher and Ebert, 1996 • Sutula et al., 1995
Kainic acid or pilocarpine-induced SE	Yes	Yes	•Synaptic reorganizations •Cell loss, gliosis •Receptor subunit changes •Memory Impairments	• Tauck and Nadler, 1985 • Ben-Ari, 1985; Covolan and Mello, 2000 • Gibbs et al., 1997; Schwarzer et al., 1997 • Kemppainen et al., 2006
Stimulation-induced SE	Yes	Yes	•Synaptic reorganizations •Cell loss, gliosis •Memory Impairments	• Sloviter, 1987 • Nissinen et al., 2000 • Kemppainen et al., 2006

generalized, based on observations from electrographic discharges and behavioral automatisms that are elicited by activation of limbic structures, followed by diffuse electrographic epileptiform discharges with accompanying behaviors defined as generalized seizures (Racine, 1972). Changes in synaptic transmission elicited by repeated kindled seizures trigger morphological reorganizations and functional deficits that lead to enhanced seizure susceptibility (McIntyre and Racine, 1986). One major advantage of the kindling model is the option to select precise time points in epileptogenesis, based on the number of stimulation-induced kindled seizures, to study the progressive cellular and molecular mechanisms that generate enhanced seizure susceptibility. Further, potential anti-epileptogenic therapeutics can be tested using this model, by aiming to block the development of kindling. However, criticisms of kindling as a model of TLE include the requirement of hundreds of stimulations (over-kindling) to produce spontaneous seizures (Löscher , 1997), which defines epilepsy.

Alternatively, SE in rodents is also a widely used model of epileptogenesis and TLE. Status epilepticus is an episode of prolonged seizure activity, without return to baseline electrographic or behavioral function between seizures. During a seizure-free latent period after SE, structural and functional neuronal changes occur, that contribute to epileptogenesis and the development of spontaneous seizures (Cavalheiro et al., 1982; Tauck and Nadler, 1985; Cronin and Dudek, 1988; Lothman et al., 1990; Leite et al., 2002). Not only is SE-induced epileptogenesis justified as a model of TLE by the latent period and occurrence of spontaneous seizures, neuropathological observations in the rat brain after SE are similar to those observed in patients with TLE (Table 1; Nadler, 1981; Ben-Ari, 1985; Tauck and Nadler, 1985; Pitkänen et al., 1998; Covolan and Mello, 2000;

Gorter et al., 2001; Chen and Buckmaster, 2005). Particularly, extensive neurodegeneration of limbic structures after SE (Nadler, 1981; Turski et al., 1983; Ben Ari, 1985; Covolan and Mello, 2000; Chen and Buckmaster, 2005), as well as aberrant axonal sprouting in the hippocampus (Tauck and Nadler, 1985; Nissinen et al., 2000), are frequent pathologies of TLE patients (Falconer et al., 1964; Margerison and Corsellis, 1966; Bruton, 1988; Sutula et al., 1989). Status epilepticus can be induced by chemoconvulsants, such as kainic (Nadler, 1981; Best et al., 1993; Sperk, 1994) or pilocarpine (Turski et al., 1983), or by electrical stimulation of the amygdala (Nissinen et al., 2000) or hippocampus (Sloviter, 1987; Lothman et al., 1990; Mazarati et al., 1998). The epileptogenic latent period can last 1 week to several months in the SE model, before the onset of spontaneous seizures (Cavalheiro et al., 1991; Hellier et al., 1998; Nissinen et al., 2000). Animals treated with kainic acid, which triggers seizures via its direct activation of glutamate receptors to induce SE, are more likely to survive and later develop spontaneous seizures compared to pilocarpine-induced SE, which may be due to more extensive neuronal damage caused by pilocarpine-induced SE (Covolan and Mello, 2000).

Neuropathology of Epileptogenesis

The use of animal models of TLE has unraveled morphological and structural alterations that may contribute to hyperexcitability of limbic structures during epileptogenesis. One of the hallmarks of such alterations is loss of hilar and CA1 interneurons in the hippocampus (Sloviter, 1987; Lowenstein et al., 1992; Best et al., 1993; Obenhaus et al., 1993; Houser and Esclapez, 1996; Morin et al., 1998; Sun et al.,

2007). Most often this is accompanied by reduced inhibitory activity (Rice et al., 1996; Cossart et al., 2001; Kobayashi and Buckmaster, 2003; Shao and Dudek, 2005; Sun et al., 2007), but unaltered or enhanced inhibition has also been observed in some areas of the epileptic hippocampus (Nusser et al., 1998; Cossart et al., 2001; Shao and Dudek, 2005). Furthermore, it is becoming apparent that the changes occurring after the initial epileptogenesis-triggering insult, leading to an imbalance between GABAergic and glutamatergic activity, do not follow a linear progression (see Straessle et al., 2003; El-Hassar et al., 2007; Rocha et al., 2007). For example, enhanced inhibition or reduced inhibition can be found in the CA1 area at different stages of epileptogenesis (El-Hassar et al., 2007). These derangements in GABAergic inhibition are not only due to loss of GABAergic neurons, but also to alterations at many levels, such as mossy fiber sprouting and the formation of aberrant recurrent excitatory circuits (Tauck and Nadler, 1985; Nissinen et al., 2000), changes in the activity of glutamate decarboxylase (Baran et al., 2004), or expression and synaptic distribution of the different subunits of $GABA_A$ receptors (Schwarzer et al., 1997; Gibbs et al., 1997; Brooks-Kayal et al.,1998; Raol et al., 2006; Zhang et al., 2007).

Despite the importance of the amygdala in TLE, much less is known about the functional or morphological changes that occur within this brain structure during epileptogenesis (Aroniadou-Anderjaska et al., 2008). In rat models of SE, the amygdala undergoes extensive degeneration; the greatest cell loss in the amygdala is observed in the anterior and posterior cortical, and medial nuclei, as well as in portions of the lateral, basolateral, and central nuclei (Tuunanen et al., 1996; Tuunanen et al., 1999). Damage in the lateral and basolateral nuclei includes a loss of GABAergic neurons (Tuunanen et al.,

1996). Likewise, impairments in GABAergic inhibitory transmission have been reported previously in the amygdala of epileptic rats (Gean et al., 1989; Smith and Dudek, 1997; Mangan et al., 2000; Benini and Avoli, 2006). In the rat BLA, spontaneous IPSPs are reduced or absent at 2 months after SE induced by hippocampal stimulation (Mangan et al., 2000), and hyperexcitable field responses are at least partly due to reduced inhibition, in epileptic rats (Smith and Dudek, 1997). However, it is not known whether inhibitory dysfunction is present in the BLA during epileptogenesis, before the onset of spontaneous seizures.

Apart from the hippocampus and amygdala, the piriform cortex and the adjacent endopiriform nucleus also undergo significant neuronal loss in animal models of epileptogenesis, and are important in the propagation of seizures (Piredda and Gale, 1985; Löscher and Ebert, 1996; Sperber et al., 1998). The piriform cortex is one of the most susceptible brain regions to damage after SE (Sperk, 1994; Covolan and Mello, 2000), and has an even faster kindling rate than the amygdala (McIntyre and Plant, 1989). The adjacent endopiriform nucleus has a low threshold for spike activity, as well as a fast kindling rate, and recently has gained attention for being important in epileptogenesis (for a review see Majak and Moryś, 2007).

Status Epilepticus and Pharmacoresistance

Status epilepticus in humans is defined as a seizure which shows no signs of arrest clinically, or without resumption of baseline central nervous system function interictally, of a duration of 5 minutes or more (Shorvon, 2001; Chen and Wasterlain, 2006). SE is an acute medical emergency associated with a high risk of mortality and

morbidity (Logroscino et al., 2002). Unlike seizures that are self-limiting, seizures during SE are enduring, and must be controlled pharmacologically as soon as possible; otherwise prolonged seizure-induced neuropathological processes can occur (Treiman, 2007). Benzodiazepines are the first-line therapy for SE; benzodiazepines allosterically modulate specific benzodiazepine-sensitive $GABA_A$ receptors to increase the efficiency of $GABA_A$ receptor-mediated inhibition (Macdonald and Olsen, 1994; McKernan and Whiting, 1996). SE becomes refractory to benzodiazepines if treatment is not administered early, (Treiman et al., 1998), and more aggressive second- and third-line antiepileptic drugs must be used (Löscher, 2007; Riss et al., 2008). After an episode of SE, patients have a high risk of developing epilepsy (Hesdorffer et al., 1998).

Similar to SE in humans, in animal models of TLE, in which SE triggers epileptogenesis, as the duration of SE increases, seizure termination with the benzodiazepine, diazepam, becomes less successful (Walton and Treiman, 1988; Jones et al., 2002), prolonged-seizure induced neuronal damage ensues (Meldrum and Horton, 1973; Corsellis and Bruton, 1983; DeGiorgio et al., 1995), and much higher concentrations of the drug are required to be effective at interrupting SE (Bleck, 1999; Jones et al., 2002). SE duration is correlated with the extent of neurodegeneration (Lemos and Cavalheiro, 1995; Gorter et al., 2003), therefore minimizing the variation of SE duration is important to maintain homogeneity within groups of rats when using animal models to study epileptogenesis. Thus, termination of SE pharmacologically is necessary to minimize variation of SE duration, but should be done at least 90 minutes after SE onset, as 90 minutes of SE is necessary to induce epileptogenesis and the development of spontaneous seizures (Lemos and Cavalheiro, 1995; Brandt et al., 2003).

Because benzodiazepines lose anticonvulsant efficacy in the first hour of SE, higher non-lethal doses are necessary to successfully terminate SE (Bleck, 1999; Jones et al., 2002).

Diazepam's weakened anticonvulsant effect with increasing duration of SE has been associated with alterations in the functional properties of $GABA_A$ receptors in the hippocampus (Kapur and Macdonald, 1997), specifically, an internalization of $GABA_A$ receptors that occurs within the first hour of SE (Goodkin et al., 2005; Naylor et al., 2005; Feng et al., 2008). Furthermore, diazepam administered early, within 2 hours after SE initiation, reduces neuronal loss in the hippocampus, but this effect is not evident if diazepam is administered 3 hours after SE initiation (Pitkänen et al., 2005). Thus, the neuroprotective potential of diazepam is reduced in the hippocampus after prolonged SE. It is not currently known whether this also occurs in other limbic regions associated with epileptogenesis and TLE that are vulnerable to seizure-induced neuronal injury.

Amygdala: Function and Anatomy

The amygdala is a limbic structure of the medial temporal lobe that is intimately associated with the neurobiology of emotional behavior. Via reciprocal connections with the cerebral cortex, the thalamus and other subcortical structures, the amygdala receives information from all sensory modalities, and plays a central role in assessing the emotional significance and modulating the consolidation of this information, as well as organizing a behavioral response (LeDoux, 1992; Davis, 1994; McGaugh et al., 1996; Fanselow and Gale, 2003; Sah et al., 2003).

The amygdala is comprised of more than ten nuclei (Figure 2; Amaral et al., 1992). The left and right amygdala are monosynaptically connected, and there are also

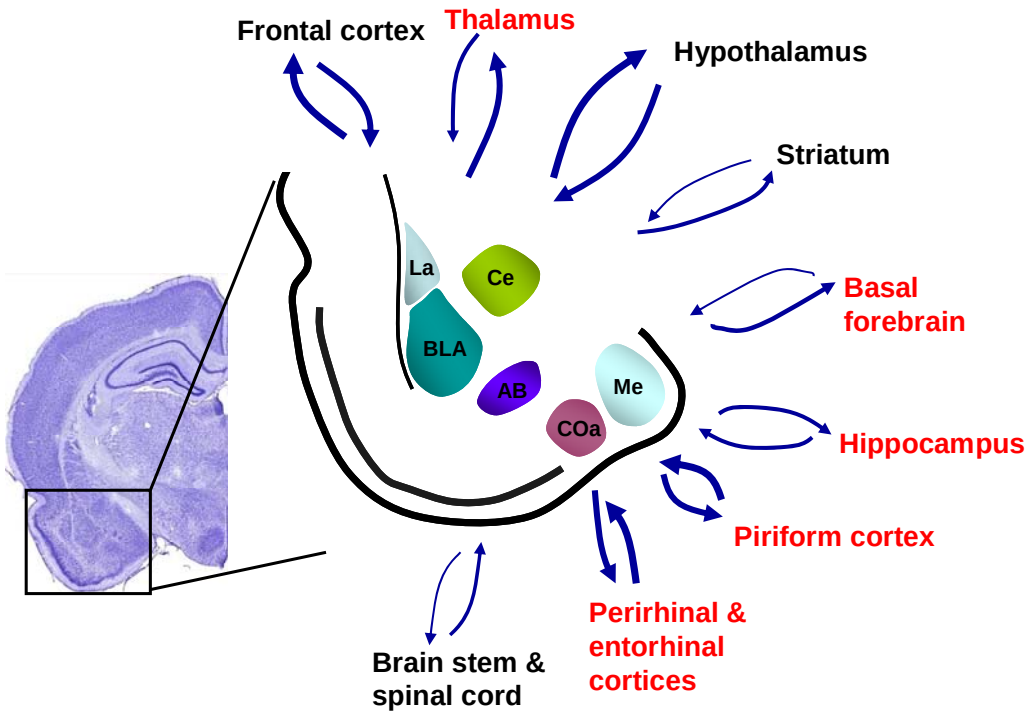

Figure 2: Anatomy of amygdala nuclei and amygdalar connections with other cortical and subcortical structures; those involved in the induction, propagation, and/or maintenance of limbic seizure activity are listed in red. Arrow thickness indicates density of connections (Pitkänen, 2000). AB, accessory basal nucleus; BLA, basolateral nucleus; Ce, central nucleus; COa, anterior cortical nucleus; La, lateral nucleus; Me, medial nucleus.

vast inter- and intra- nuclear connections between and within the amygdala nuclei (Ottersen, 1982). Tract-tracing studies indicate that information coming into the amygdala follows a lateral to medial flow, from the basolateral complex to the main output group, the centromedial amygdala (Sah et al., 2003). The basolateral amygdaloid complex is comprised of the lateral, basolateral, and the accessory basal nuclei (Krettek and Price, 1978). The basolateral nucleus (BLA) of the basolateral complex is further segregated into magnocellular, intermediate, and parvocellular divisions (Amaral et al., 1992), distinguished by large, medium, and small sized neuronal morphologies, respectively; connections within and between each of these divisions also exist. The BLA receives input from all sensory modalities, as well as from memory and autonomic systems. Via the external capsule, the BLA receives excitatory input from the frontal, parietal, and olfactory cortices, the hippocampus, hypothalamus, and thalamus (Fig 2; Amaral et al., 1992; Sah et al., 2003). Projections from the BLA to the frontal cortex and hippocampus are more extensive than from any of the other amygdaloid nuclei (Amaral et al., 1992). Other extra-amygdaloid outputs from the BLA also include projections to the thalamus, striatum, and nucleus accumbens (Sah et al., 2003).

Unlike the structural organization of hippocampal pyramidal neurons and interneurons, neurons in the BLA are randomly situated (McDonald, 1984). Glutamatergic, excitatory neurons are the most prominent cell type in the BLA and are, accordingly, also called principal neurons. Principal neurons in the BLA are pyramidal in shape with numerous dendritic spines, or semi-pyramidal in shape and sparsely spinous (Tosevski et al., 2002). GABAergic inhibitory interneurons are aspiny, are spherical or fusiform in shape, and have slightly smaller soma compared to principal

neurons (McDonald, 1982; Smith & Pare, 1994; Tosevski et al., 2002). The axons of pyramidal cells have substantial local collaterals, which target local interneurons and other nearby pyramidal cells (Smith et al., 2000). Additionally, interneurons are interconnected locally, forming a local network of synaptic activity (Lang and Pare, 1998). Principal neurons in the BLA can also be distinguished from interneurons electrophysiologically, whereby principal neurons fire a single spike and then accommodate in response to a depolarizing pulse, while interneurons in response to the same depolarizing pulse fire a non-accommodating high-frequency spike discharge (Washburn and Moises, 1992). Additionally, principal neurons in the BLA are further electrophysiologically discernible from interneurons by the presence of the hyperpolarization-activated cation current, produced by hyperpolarization-activated and cyclic-nucleotide-gated ion channels (Mahanty and Sah, 1998; Park et al., 2007).

Several populations of interneurons have been described in the BLA, based on the expression of neuropeptides or calcium binding proteins. Parvalbumin-expressing interneurons make up about 50% of the interneuron population in the BLA, often co-localizing with calbindin (McDonald and Betette, 2001). Other populations of interneurons in the BLA include somatostatin-expressing interneurons (McDonald and Mascagni, 2002), those expressing cholecystokinin and either calretinin or vasoactive intestinal peptide, and those expressing cholecystokinin but not calretinin or vasoactive intestinal peptide (Mascagni and McDonald, 2003).

Amygdala and Temporal Lobe Epilepsy

Is the amygdala important in epileptogenesis and epilepsy? Although a frequent pathology observed in TLE is hippocampal sclerosis (Margerison and Corsellis, 1966; Babb and Brown, 1987; Bruton, 1988), neuronal loss and gliosis in the amygdala is also reported in a significant subpopulation of patients (Margerison and Corsellis, 1966; Bruton, 1988; Armstrong, 1993; Hudson et al., 1993). Overall, it is estimated that 30-50% of drug-refractory TLE patients have amygdala damage (Margerison and Corsellis, 1966; Bruton, 1988); isolated amygdala damage has also been observed (Miller et al., 1994). Furthermore, either the amygdala or the hippocampus is most often the focus of ictal onset in temporal lobe seizures (Quesney, 1986; Isokawa-Akesson et al., 1987; Dewar et al., 1996; Pitkänen et al., 1998). Thus, temporal lobectomy is often a necessary procedure when anti-epileptic drugs fail to control seizures in drug-refractory TLE (Babb and Brown, 1987), and amygdalectomy alone is sufficient to eliminate seizures in some cases (Feindel and Rasmussen, 1991; Jooma et al., 1995). Since the amygdala modulates cognitive functions and plays an essential role in emotional behavior (LeDoux, 1992; Davis, 1994; McGaugh et al., 1996; Fanselow and Gale, 2003; Sah et al., 2003) and affective disorders (Drevets, 1999; Rauch et al., 2000; Chen et al., 2005), as well as in sexual behavior (Kostarczyk, 1986; Salamon et al., 2005), amygdala dysfunction in TLE is important not only for its role in the generation of seizures, but also for its role in the behavioral, cognitive (Kanner, 2006; Swinkels et al., 2006; Briellmann et al., 2007; Richardson et al., 2007), and sexual (Herzog et al., 2003; Harden, 2006) dysfunctions that often are reported in epilepsy patients.

The role of the amygdala in epileptogenesis and TLE may be even more significant than the hippocampus (for a review see Aroniadou-Anderjaska et al., 2008). The amygdala requires a lower stimulation threshold and fewer stimulations to generate kindled seizures, compared to the hippocampus (Goddard et al., 1969; McIntyre and Racine, 1986). Particularly, the BLA is important in the initiation and spread of seizure activity. The BLA is responsible for the generation and spread of status epilepticus, even when seizures are evoked in extra-amygdaloid regions (White and Price, 1993a,b), and has a lower stimulation threshold for inducing status epilepticus than other amygdala nuclei or the piriform cortex (Mohapel et al., 1996). In amygdalar slices from epileptic rats, the BLA is hyperexcitable, with multiple action potential bursts (Mangan et al., 2000) and reduced spontaneous inhibitory post-synaptic potentials (Smith and Dudek, 1997; Mangan et al., 2000). The BLA is also more prone to the generation of epileptiform activity when compared to the lateral nucleus of the amygdala in the epileptic rat brain (Nittykoski et al., 2004). Nevertheless, there is limited knowledge on the pathological and pathophysiological alterations that occur in the BLA during epileptogenesis.

GluRK5, Amygdala, and Epilepsy

Kainate receptors are one of the three subtypes of ionotropic glutamate receptors, and are tetramers made from five subunits: GluR5, GluR6, GluR7, KA1, and KA2. GluR5, GluR6, and GluR7 are low affinity subunits that form functional homomeric receptors, as well as heteromeric receptors with other subunits (Bettler et al., 1990; Sommer et al., 1992; Egebjerg and Heinemann, 1993), while the higher affinity KA1 and

KA2 subunits can only form functional subunits when expressed as heteromers with the GluR5, GluR6, or GluR7 subunits (Herb et al., 1992; Schiffer et al., 1997).

Kainate receptors mediate fast excitatory synaptic transmission (Castillo et al., 1997; Cossart et al., 1998), modulate transmitter release at both excitatory and inhibitory synapses, and are involved in short- and long-term synaptic plasticity mechanisms (Huettner, 2003; Lerma, 2003). *In situ* hybridization studies have shown that mRNA levels of GluR5, GluR6 and KA2 subunits are highly expressed in the amygdala (Braga et al., 2003). Particularly, the GluR5 subunit is higher in the amygdala than in other brain regions, and is concentrated in the BLA and medial nuclei (Braga et al., 2003). Therefore, GluR5-containing kainate receptors (GluR5KR) may play a prominent role in both physiological and pathological conditions of this brain region.

The balance between excitation and inhibition in the brain is maintained by a number of mechanisms, including neurotransmitters and neuromodulators acting on somatodendritic receptors, where they mediate or modulate neuronal responses to synaptic input, or on receptors at presynaptic terminals, where they directly modulate neurotransmitter release. In the BLA, GluR5KRs that are present on somatodendritic areas of both excitatory and inhibitory neurons contribute to synaptic transmission; GluR5KRs on GABAergic presynaptic terminals modulate GABA release (Braga et al., 2003; Braga et al., 2004). It has been shown previously that the specific GluR5KR agonist, ATPA, enhances the frequency and amplitude of spontaneous GABAergic currents (sIPSCs) recorded from BLA pyramidal cells in the BLA *in vitro* (Braga et al., 2003), suggesting that GluR5KR activation depolarizes inhibitory interneurons. GluR5KRs are also present on GABAergic terminals contacting pyramidal cells, and

activation of these receptors bidirectionally modulates the release of GABA, such that low concentrations ATPA enhanced GABA release at interneuron to pyramidal cell synapses, while high concentrations depressed it (Braga et al., 2003).

Recently, an interest in the GluR5KRs, which are highly expressed in the amygdala compared to the hippocampus (Bettler et al., 1990; Li et al., 2001; Braga et al., 2003), has emerged as being important in epileptogenesis and epilepsy (Aroniadou-Anderjaska et al., 2007). Activation of GluR5KRs by the agonist ATPA in the BLA can induce epileptiform discharges *in vitro* (Rogawski et al., 2003), or seizure activity by systemic or intra-amygdaloid injections *in vivo* (Rogawski et al., 2003; Kaminski et al., 2004). GluR5KR antagonists can prevent the induction of limbic seizures induced *in vivo* by pilocarpine or by electrical stimulation (Smolders et al., 2002). Evidence that GluR5KRs play an important role in TLE comes from the findings that there are alterations in both the function of GluR5KRs (Kortenbruck et al., 2001; Palma et al., 2002) and the expression of the GluR5 subunit in TLE patients (Mathern et al., 1998) and epileptic rats (Ullal et al., 2005). However, it is not known if the function of GluR5KRs is altered during epileptogenesis, in the BLA.

Summary

Understanding the nature of the structural and functional alterations that lead to the development of epilepsy is crucial for the discovery of disease modifying therapies that can inhibit epileptogenesis, or reduce the severity of the developing disease. Despite the importance of amygdala in the pathogenesis of TLE, little is known about the

pathological and pathophysiological changes that occur in this brain structure during epileptogenesis.

Hypothesis: Neuropathological and pathophysiological alterations occur in the amygdala, in particular the basolateral nucleus of the amygdala, after kainic acid-induced SE, that may contribute to epileptogenesis.

Aim 1: To determine if GABAergic inhibitory function is impaired in the basolateral amygdala during epileptogenesis. Because a derangement in the GABAergic system is often observed in hyperexcitable, epileptic circuits, we used the kainic acid-induced SE model of TLE to study inhibitory function in the BLA during epileptogenesis. By recording spontaneous inhibitory post-synaptic currents (IPSCs) and miniature (IPSCs) from BLA pyramidcal cells (Figure 3) at 7-10 days after kainic acid-induced SE, we investigated alterations in the GABAergic system in the BLA during epileptogenesis. We also used design-based stereology to quantify the total number of neurons remaining in the BLA, as well as the number of GABAergic neurons remaining in the BLA, at 7-10 days after kainic acid-induced SE, compared to sham controls.

Aim 2: To examine the extent of neurodegeneration in limbic structures during epileptogenesis after kainic acid-induced SE, and to determine if administration of diazepam after prolonged SE reduces this neurodegeneration. Neuroprotection after prolonged SE has important implications in epileptogenesis, not

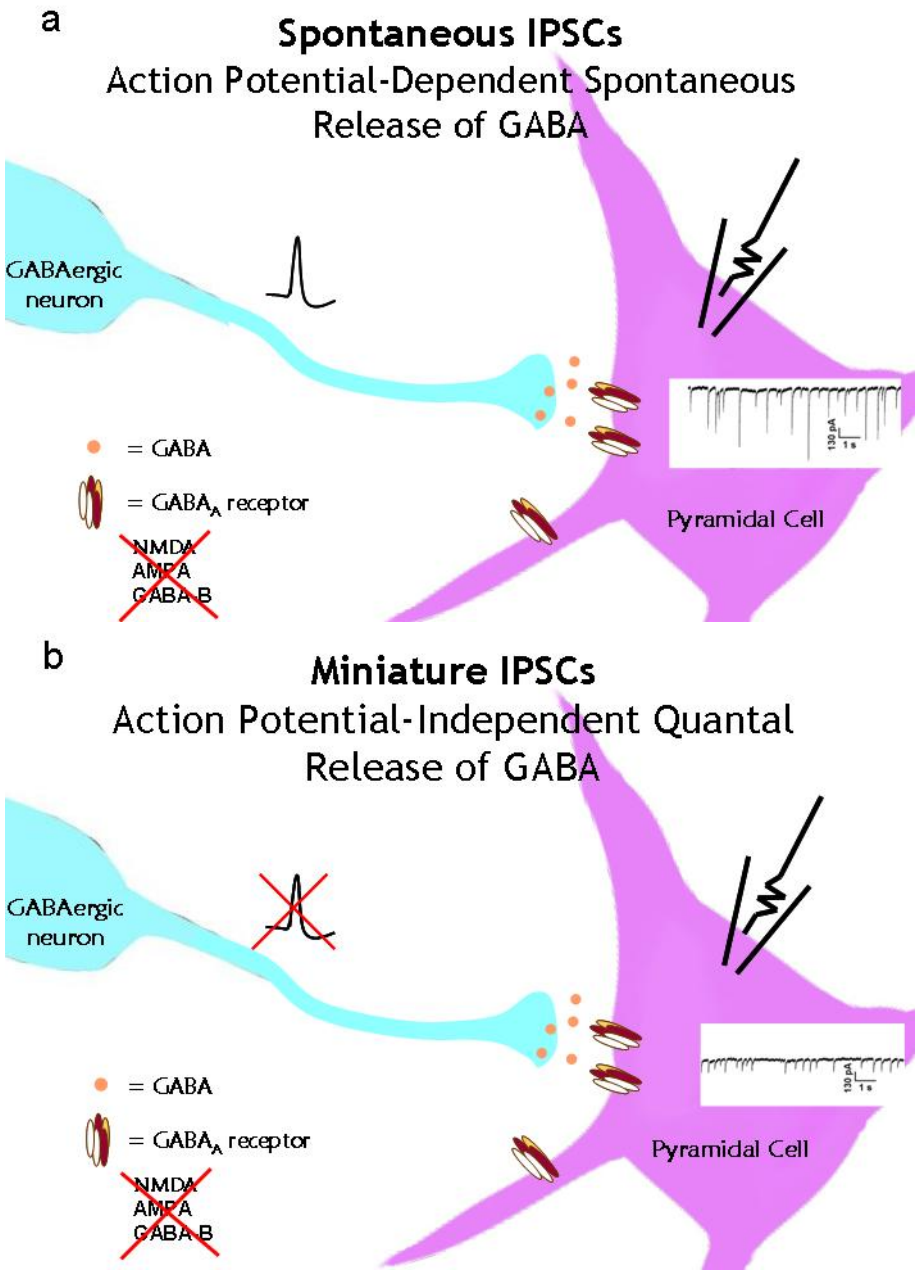

Figure 3. Spontaneous and minitiature inhibitory post-synaptic currents (IPSCs). **(a)** To identify alterations in overall tonic inhibition in the BLA during epileptogenesis, we measured spontaneous GABA release by recroding spontaneous IPSCs from BLA pyramidal cells at 7-10 days after kainic acid-induced SE. **(b)** To identify alterations in the quantal release of GABA in the BLA during epileptogenesis, we recorded miniature IPSCs from BLA pyramidal cells at 7-10 days after kainic acid-induced SE.

only for disease modification, but also for preventing subsequent neurological dysfunction, such as the cognitive deficits and/or mood disorders observed in some TLE patients (Benuzzi et al., 2004; Beyenburg 2005; Kondziella, 2007). We quantified degenerating neurons from brain sections 7-9 days after SE with Fluoro-Jade C, a fluorescent compound that selectively binds to degenerating neurons, to compare the extent of neurodegeneration in limbic structures that are important in epileptogenesis and TLE, and to assess the neuroprotective efficacy of SE termination with diazepam.

CHAPTER 2

Section: Cellular/Molecular

GABA-mediated inhibition in the basolateral amygdala following status epilepticus: selective loss of GABA neurons and impaired inhibitory function

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Abbreviated title: GABA inhibition in the BLA following status epilepticus

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Abstract

The basolateral amygdala (BLA) plays a central role in mesial temporal lobe epilepsy (MTLE) yet little is known about the functional changes within the nucleus that accompany epileptogenesis. Here we investigated GABA-mediated inhibition in the rat BLA following kainate-induced status epilepticus, a model of MTLE. The experiments were conducted 7 to 10 days after status epilepticus, when epileptiform discharges were apparent on the EEG, but before the occurrence of spontaneous motor seizures. Whole-cell recordings from BLA pyramidal neurons in brain slices showed a reduction in the frequency and amplitude of action potential-dependent spontaneous IPSCs, and a reduced frequency but not amplitude of miniature IPSCs. In addition, there was impairment in GluR5 kainate receptor-modulation of GABAergic synaptic activity, which ordinarily maintains inhibitory tone within the BLA. Design-based stereology demonstrated a 15% reduction in total neuronal counts in the BLA of rats that had experienced status epilepticus, but there was a 43% decrease in GAD67 immunoreactive GABA neurons, indicating that these neurons are selectively vulnerable. Fluoro-Jade C staining showed ongoing neuronal degeneration. There was also a reduction in GluR5 subunit expression as revealed by Western blot analysis, consistent with defective GluR5 receptor function. In the status epilepticus model of MTLE, selective loss of GABA inhibitory neurons and impaired function of surviving GABA neurons leads to reduced inhibitory function, which contributes to hyperexcitability and a propensity toward epileptiform activity and seizure discharges.

Introduction

The epilepsies are episodic neurological disorders characterized by the occurrence of recurrent seizures. In a significant proportion of patients, epilepsy follows an acute brain insult such as traumatic brain injury, stroke, or a period of prolonged and intense seizures (status epilepticus). When an acute brain insult is the etiological factor, the symptoms of epilepsy often appear after a seizure-free latent period following the acute injury (Dudek et al., 2002). During this seizure-free period, which can vary from months to years (French et al., 1993; Mathern et al., 1995), neuronal networks in certain brain regions undergo structural and functional changes that lead to hyperexcitability and eventually to the expression of spontaneous seizures; this process is referred to as epileptogenesis (Dalby and Mody, 2001).

In mesial temporal lobe epilepsy (MTLE), the most common type of epilepsy in adults (Benbadis, 2001; Engel, 2001), the hippocampus and the amygdala play central roles, and either of them, or both, can be considered epileptic foci (Quesney, 1986; Goldring et al., 1992; Gotman and Levtova, 1996; Bragin et al., 2005; Bragin et al., 2007). A variety of animal models have been used to study the pathophysiology of MTLE. One of the most common is the "post-status epilepticus model" in which epilepsy develops over the course of days to weeks after a sustained period of intense seizure activity (White, 2002; Leite et al., 2002). During the latent period before the appearance of spontaneous behavioral seizures, epileptiform events are observed in the electroencephalogram (EEG) and neurons in the CA1 region and the dentate gyrus of the hippocampus display signs of hyperexcitability such as increased bursting in response to

stimulation (Mangan and Bertram, 1998; Kobayashi and Buckmaster, 2003; Sloviter et al., 2006; El-Hassar et al., 2007).

A great deal of information has been accumulated on the structural and functional changes in the hippocampus that accompany epileptogenesis in this model. Many investigators have observed a loss of interneurons (Sloviter, 1987; Lowenstein et al., 1992; Best et al., 1993; Obenhaus et al., 1993; Houser and Esclapez, 1996; Morin et al., 1998; Sun et al., 2007), most often accompanied by reduced inhibitory activity (Rice et al., 1996; Hirsch et al., 1999; Cossart et al., 2001; Kobayashi and Buckmaster, 2003; Shao and Dudek, 2005; Sun et al., 2007). Unaltered or enhanced inhibition have also been observed in some areas of the epileptic hippocampus (Nusser et al., 1998; Cossart et al., 2001; Shao and Dudek, 2005; Sloviter et al., 2006). Overall, however, there is substantial evidence that the hyperexcitability of epileptic hippocampal networks in post-status epilepticus models results from an imbalance between excitatory glutamatergic and inhibitory GABAergic synaptic drive such that GABA-mediated inhibition is relatively weakened. The imbalance appears to evolve during the latent period and precede the appearance of overt behavioral seizures (El-Hassar et al., 2007).

Despite the importance of the amygdala in MTLE, there is little information on functional changes that occur within this brain structure during epileptogenesis (Aroniadou-Anderjaska et al., 2008). In the present study, we investigated the consequences of kainic acid (KA)-induced status epilepticus on GABA neurons and GABA-mediated inhibitory function in the amygdala of young adult rats. We focused on the basolateral nucleus, which, of all the amygdala nuclei, plays the most important role in the initiation and spread of seizures (White and Price, 1993a, 1993b; Mohapel et al.,

1996). We found a loss of GABA neurons as well as defective function of GluR5 kainate receptors on the surviving GABA neurons, which together may contribute to hyperexcitability in the nucleus.

Materials and Methods

Animals. Experiments were performed on male Sprague-Dawley rats (Taconic Farms, Rockville, MD), 5–6 weeks old, weighing 170–200 g at the start of the experiments. Animals were individually housed in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care, in an environmentally controlled room (20–23°C, 12-h light/12-h dark cycle, lights on 07:00 a.m.), with food and water available *ad libitum*. All animal use procedures were in accordance with the National nstitutes of Health *Guide for the Care and Use of Laboratory Animals*, and were approved by the Animal Care and Use Committees of the National Institute of Neurological Disorders and Stroke and Uniformed Services University of the Health Sciences.

Surgery. Following 5 days of acclimatization, the rats were stereotaxically implanted with 5 cortical stainless steel screw electrodes under general anesthesia (ketamine 60 mg/kg intraperitonally (i.p.), medetomidine 0.5 mg/kg i.p.), using the following coordinates, in mm, after Paxinos and Watson (1998): Two frontal electrodes AP = +1.5, ML = ±2.5 from bregma; two parietal electrodes AP = –5.0, ML = ±4 from bregma; and a single cerebellar reference electrode midline, AP = –1.0 from lambda. Each screw electrode with socket (E363/20; Plastics ONE Inc., Roanoke, VA) was placed in a plastic

pedestal (MS363; Plastics ONE Inc., Roanoke, VA) and attached to the skull with dental acrylic cement. Anesthesia was reversed by i.p. injection of 1 mg/kg atipamezol.

Induction of status epilepticus. After 1 week of recovery, 25 rats were injected (i.p.) with KA to induce status epilepticus ("KA-SE rats"). Fifteen implanted rats were injected with corresponding amounts of saline at corresponding times as the KA-treated rats; this group of rats served as controls ("sham control rats"). Status epilepticus was induced following a modified titration protocol after Hellier et al. (1998). In order to maximize consistency of the status epilepticus expression between rats, EEG seizure activity rather than the behavioral seizure correlate was used to determine the necessity of additional KA injections. Rats were initially treated with 7.5 mg/kg KA (i.p.) dissolved in 0.1 M phosphate buffered normal saline (PBS; 5mg/ml), followed by additional 5 mg/kg doses every 60 min, until status epilepticus began, defined by 5 min of continuous generalized electrographic seizure activity (all four cortical electrodes involved; no breaks longer than 10 sec). Following this protocol, rats received between 12.5 and 32.5 mg/kg KA. Status epilepticus was allowed to continue without intervention for 180 min, and then 25–30 mg/kg of diazepam was injected i.p. for termination of SE. Fluid was substituted with 6 ml lactated Ringer's solution injected subcutaneously (s.c.) after resolution of continuous convulsive seizures, usually less than 10 min after the diazepam injection. The sham control rats also received diazepam injections. For three days after SE, rats were offered fruits and soft chow. Additional lactated Ringer's solution was administered by s.c. injection if dehydration was apparent. The wooden bedding was changed to iso-PADs^{TM} (Harlan Teklad, Madison, WI) to avoid aspiration in case of frequent seizures.

EEG recordings and analysis. Recordings were performed in freely moving rats using a commercially available Stellate® EEG monitoring system modified for the use in rodents (Respitech Medical Inc., Lancaster, PA; sampling rate, 200 Hz). EEG recordings were visually analyzed offline with filter settings set to 0.3 Hz low frequency filter, 60 Hz notch filter, and 70 Hz high frequency filter, using the Harmonie Viewer 6.1c from Stellate® (Montreal, Quebec, Canada). An electrographic seizure was defined as a period of EEG changes marked by an abrupt starting and ending, that included a minimum of 10 sec of consistent, repetitive discharges at least double the amplitude of the background activity and not less than 1 Hz frequency. A single graphoelement was classified as sharp wave when it was clearly distinguishable from movement artifacts, had twice the amplitude of the background EEG, and was less than 200 ms in duration. Animals were recorded starting in the early morning, throughout SE, and continuing for at least two hours after the diazepam injection (7 to 9 hours total recording time per animal). The EEG seizure activity had stopped at the end of the recording period in 20 of the 25 KA-treated rats, with four mortalities.

Post-status epilepticus EEG monitoring for detection of spontaneous sharp waves and electrographic seizure activity was performed either for 24 hours, 6 to 9 days after status epileptics (n = 16 rats) or on days 6 and 7 for 8 hours per day (n = 5 rats). The non-occurrence or occurrence of spontaneous sharp waves, spikes and seizures was noted during each recording period.

Amygdala slice electrophysiology. Coronal slices containing the amygdala were prepared from rats, at 7 to 10 days after status epilepticus. The rats were anesthetized with CO_2 and then decapitated. The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 22 glucose, bubbled with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. A block containing the amygdala region was prepared, and 400 µm slices were cut with a Vibratome (Series 1000; Technical Products International, St. Louis, MO). Slices were kept in a holding chamber containing oxygenated ACSF at room temperature, and recordings were initiated ≥1 hr after slice preparation. For whole-cell recordings, slices were transferred to a submersion-type recording chamber, where they were continuously perfused with oxygenated ACSF, at a rate of 3–4 ml/min. Neurons were visualized with an upright microscope (Nikon Eclipse E600fn; Nikon, Tokyo, Japan) using Nomarski-type differential interference optics through a 60× water immersion objective. All experiments were performed at room temperature (28 °C). Tight-seal (>1 GΩ) whole-cell recordings were obtained from the cell body of pyramidal-shaped neurons in the BLA region. Patch electrodes were fabricated from borosilicate glass and had a resistance of 1.5–5.0 MΩ when filled with a solution containing (in mM): 135 Cs-gluconate, 10 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, 10 HEPES, 2 Na-ATP, 0.2 Na_3GTP, pH 7.3 (285–290 mOsm). Neurons were voltage-clamped using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). IPSCs were pharmacologically isolated and recorded at a –70 mV holding potential. Access resistance (5–24 MΩ) was regularly monitored during recordings, and cells were rejected if it changed by >15% during the experiment. The signals were filtered at 2 kHz, digitized (Digidata 1322A; Axon

Instruments), and stored on a computer using pClamp9 software (Axon Instruments). The peak amplitude, 10–90% rise time, and decay time constant of IPSCs were analyzed off-line using pClamp9 software and the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). Miniature IPSCs (mIPSCs) were analyzed off-line using the Mini Analysis Program and detected by manually setting the mIPSC threshold (~1.5 times the baseline noise amplitude) after visual inspection.

Extracellular recordings of evoked and spontaneous field potentials were obtained from the BLA, while stimulus pulses were applied to the external capsule. Recording electrodes were filled with ACSF, and had a resistance of 4 to 7 MΩ.

Western blotting. Six rats treated with KA as described above to induce status epilepticus and three sham control rats were used for measuring the protein expression levels of the GluR5 kainate receptor subunit and the α1 subunit of the $GABA_A$ receptor in the BLA. The CA3 subfield of the hippocampus and the ventral posteromedial and ventral posterolateral (VPM/VPL) thalamic nuclei were also included in the Western blot analysis for comparison; previous studies have found moderate and weak expression of the GluR5 subunit in the CA3 area and the VPM/VPL nuclei, respectively (Li et al., 2001; Braga et al., 2003). Following EEG monitoring on the sixth to ninth day after status epilepticus, rats were anesthetized with CO_2 and then decapitated. Discrete brain regions were microdissected from individual 800 μm-thick brain sections cut with a Vibratome (Technical Products International). The tissues were sonicated in lysis buffer (1% NP-40, 20 mM Tris, pH 8.0, 137 nM NaCl, 10% Glycerol, Tyr & Ser/Thr Phosphatase Inhibitor Cocktails; Upstate, Temecula, CA). After removal of cellular debris by centrifugation,

protein levels in the lysates were measured by the Bradford Coomassie Blue colorimetric assay (Bio-Rad Laboratories, Hercules, CA) and equalized accordingly. Aliquots (60 µg) were boiled for 5 min in the presence of loading buffer (NuPAGE LDS Sample Buffer (4x); Invitrogen, Carlsbad, CA), then placed on ice for 1 min. Each brain region was loaded in triplicate and proteins were separated on a 7.5% SDS-PAGE under reducing conditions using the Bio-Rad Mini-Protean3 cell system. Proteins were transferred to nitrocellulose membranes (0.45µm; Invitrogen). After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 1 hour, blots were incubated overnight at 4 °C with specific primary antibodies: anti-GluR5, 1:500 (Tocris Bioscience, Ellisville, Missouri), and anti-$GABA_A$ α1 (1:200, Chemicon), prepared in 5% bovine serum albumin (BSA) in TBS-T. After washing in TBS-T, membranes were incubated with peroxidase-conjugated goat anti-rabbit (1:10,000; Jackson Immuno Research, West Grove, PA) prepared in 5% BSA in TBS-T for 2 hours, at room temperature. Anti-β-actin (1:500; Cell Signaling Technology, Danvers, MA) was used as a loading control in all experiments. After washing in TBS-T, blots were developed using enhanced chemilluminescence detection according to the manufacture's recommendation (Pierce, Rockford, IL) and exposed to BioMax MR Film (Kodak Biomax, Rochester, NY) under non-saturating conditions. The blots were stripped with Restore Western blot Stripping Buffer (Pierce) and then incubated with subsequent antibodies (see above). Absorbance values of bands for GluR5 and $GABA_A$ α1 were analyzed by densitometry using Image J analysis systems (NIH, Bethesda, MD), and normalized relative to the β-actin absorbance value.

Histological studies. Five rats treated with KA as described above to induce status epilepticus and five sham control rats were used for histological studies of the BLA. Six to 9 days after the day of status epilepticus, rats were EEG monitored for 24 hours. On the day after monitoring, the animals were deeply anesthetized using ketamine (60 mg/kg i.p.) and medetomidine (0.5 mg/kg i.p.) and transcardially perfused with PBS (100 mL) followed by 4% paraformaldehyde (250 mL). The brains were removed and post-fixed overnight at 4º C, then transferred to a solution of 30% sucrose in PBS for 72 hours, and frozen with dry ice before storage at –80º C until sectioning. A 1-in-6 series of sections containing the rostro-caudal extent of the BLA was cut at 40 µm on a sliding microtome. One series of sections was mounted on slides (Superfrost Plus; Daigger, Vernon Hills, IL) in PBS for Nissl staining with cresyl violet. An adjacent series of sections was also mounted on slides for Fluoro-Jade C staining. The remaining series of sections were placed in a cryoprotectant solution (30% ethylene glycol and 30% glycerol in 0.05 M sodium phosphate buffer) and stored at –20º C until processing for immunohistochemistry.

Fluoro-Jade C staining. Fluoro-Jade C (Histo-Chem, Jefferson, AK) was used to identify dying neurons in the BLA, at 7–10 days after SE. Mounted sections were air-dried overnight, then immersed in a solution of 1% sodium hydroxide in 80% ethanol for 5 min. The slides were then rinsed for 2 min in 70% ethanol, 2 min in dH_20, and incubated in 0.06% potassium permanganate solution for 10 min. After a 2 min rinse in dH_20, the slides were transferred a 0.0001% solution of Fluoro-Jade C dissolved in 0.1% acetic acid

for 10 min. Following three 1-min rinses in dH_20, the slides were dried on a slide warmer, cleared in xylene for at least 1 min and coverslipped with DPX (Sigma).

GAD67 immunohistochemistry. To stain GAD67-immunoreactive neurons, a l-in-6 series of free-floating sections was collected from the cryoprotectant solution, washed three times for 5 min each in 0.1 M phosphate buffered saline (PBS), then incubated in a blocking solution containing 10% normal goat serum (NGS; Chemicon), and 0.5% Triton X-100 in PBS for one hour at room temperature. The sections were then incubated with mouse anti-GAD67 serum (1:1000, MAB5406; Chemicon), 5% NGS, 0.3% Triton X-100, and 1% bovine serum albumin, overnight at 4º C. After rinsing three times in 0.1% Triton X-100 in PBS, the sections were incubated with Cy3-conjugated goat anti-mouse antibody (1:1000; Jackson ImmoResearch) and 0.0001% DAPI (Sigma, St. Louis, MO) in PBS for one hour at room temperature. After a final rinse in PBS, sections were mounted on slides, air dried for 30 min, then coverslipped with ProLong Gold antifade reagent (Invitrogen).

Stereological quantification. Design-based stereology (see Appendix for a description of stereological methodology) was used to quantify total number of neurons on Nissl-stained sections and total number of inhibitory neurons on GAD67-stained sections in the BLA of KA-SE and sham control rats. Sections were viewed with a Zeiss Axioplan 2ie (Oberkochen, Germany) fluorescent microscope with a motorized stage, interfaced with a computer running StereoInvestigator 7.5 (MicroBrightField, Williston, VT). The BLA was identified on slide-mounted sections and delineated under a 2.5× objective, based on

the atlas of Paxinos and Watson (1998). Estimated totals were determined using the optional fractionator probe, and all sampling was done under a 63× oil immersion objective.

For Nissl-stained neurons in the BLA of both KA-SE and sham control rats, a 1-in-6 series of sections was analyzed (on average, 6 sections). The counting frame was 35 × 35 µm, the counting grid was 190 × 190 µm, and the disector height was 12 µm. Nuclei were counted when the top of the nucleus came into focus within the disector which was placed 2 µm below the section surface. Section thickness was measured at every counting site, and the average mounted section thickness was 19 µm. An average of 288 Nissl-stained neurons per rat was counted.

For GAD67-stained neurons in BLA, of both KA-SE and sham control rats, a 1-in-6 series of sections was analyzed (on average, 6 sections), the counting frame was 60 × 60 µm. The counting grid was 100 × 100 µm for KA-SE rats and 110 × 110 µm for sham control rats. The disector height was 12 µm (n = 1), 16 µm (n = 5), or 20 µm (n = 4), depending on extent of antibody penetration in the tissue. Cells were counted if they came into focus within the disector, which was placed 2 µm below the section surface. Section thickness was measured at every fifth counting site, and the average mounted section thickness was 40 µm. An average of 207 GAD67-stained neurons per rat was counted. The coefficient of error (CE) for the estimated total of Nissl-stained and GAD67-stained neurons in the BLA was calculated using both the Gunderson (m = 1; Gundersen et al., 1999) and Schmitz-Hof (2nd Estimation; Schmitz and Hof, 2000) equations.

Quantification of Fluoro-Jade C. Tracings of the BLA from an adjacent series of Nissl-stained sections were superimposed on the sections stained with Fluoro-Jade C. Fluoro-Jade C positive cells were counted at 20×, and recorded as number of cells per section from, on average, 6 sections containing the BLA of sham control rats and KA-SE rats.

Statistical analysis. All statistical values are presented as mean ± SEM. Results from sham control and KA-SE groups were compared using the unpaired Student's *t* test; $p < 0.05$ was considered statistically significant. Sample sizes (n) refer to the number of rats, except for the electrophysiology results where "n" refers to the number of slices.

Drugs. The following drugs were used: kainic acid (Tocris Cookson, Ballwin, MO), diazepam (Hospira Inc., IL), D-APV (Tocris; an NMDA receptor antagonist), (+)-(2*S*)-5,5-dimethyl-2-morpholineacetic acid (SCH50911; Tocris; a $GABA_B$ receptor antagonist), ATPA (Sigma; a GluR5 agonist; see Clarke et al., 1997), GYKI 52466 (Tocris; an AMPA receptor antagonist), and TTX (Sigma; a sodium channel blocker).

Results

Status epilepticus

Status epilepticus by EEG and behavioral criteria occurred in 24 out of 25 rats treated with KA. One rat experienced only focal seizures with unilateral forelimb clonus and was not studied. None of the saline-treated sham control rats displayed EEG or behavioral seizures during the observation period (n=19). Diazepam (25–30 mg/kg) was administered 180 min after the beginning of status epilepticus in the KA-treated rats to

terminate the behavioral and EEG seizure activity. The mean cumulative duration of EEG seizure activity in the KA treated rats was 257.1 ± 13.9 min (n=23). One rat died during status epilepticus, and three rats died after diazepam treatment.

EEG indicators of epileptogenesis

In the post-status epilepticus model as conducted according to the protocol we used, a high proportion of animals eventually develop spontaneous motor seizures (Hellier et al., 1998). However, to separate the effects of chronic seizures from the changes related to epileptogenesis, we studied animals in the latent period before the development of overt behavioral seizures. Thus, all experiments were conducted with animals 6 to 10 days after status epilepticus, at a time that they had recovered from the initial insult but before the occurrence of spontaneous motor seizures. These animals had clear evidence of epileptogenesis based on the presence of abnormal epileptiform discharges in the EEG. Thus, in all rats that survived KA-induced status epilepticus, we detected spontaneous sharp waves and/or spike wave complexes in recordings carried out 6 to 9 days after the episode of status epilepticus (Fig. 1A, B) (n=20). Spontaneous EEG seizures and/or episodes of periodic generalized epileptic discharges (Fig. 1B, C) occurred in 55 % of the rats (n=11).

Alterations in $GABA_A$ receptor-mediated IPSCs

To investigate alterations in GABAergic transmission in the rat BLA, 7–12 days after SE, we recorded action potential-dependent, spontaneous IPSCs (sIPSCs) and miniature IPSCs (mIPSCs) from the somata of BLA pyramidal-shaped neurons of KA-SE rats and

sham control rats. sIPSCs were recorded at a holding potential of −70 mV, and in the presence of D-APV (50 μM), GYKI 52466 (50 μM), and SCH50911 (20 μM) to block NMDA, AMPA, and $GABA_B$ receptors, respectively. The mean frequency of sIPSCs was 1.9 ± 0.7 Hz (n = 23) in sham control rats, and 1.1 ± 0.4 Hz in KA-SE rats, 41.2 ± 5.1 % lower than in the sham group (n = 21; $p < 0.01$; Fig. 2). The mean amplitude of sIPSCs in KA-SE rats was also reduced to 71.0 ± 4.8% of the control value (n = 21; $p < 0.01$; Fig. 2). There were no significant differences between KA-SE rats and sham control rats in the rise time and the decay time constant of the sIPSCs. These results suggest a reduced inhibitory tone of BLA pyramidal cells on days 7 to 10 after KA-SE.

To determine whether the reduction of sIPSCs was associated with a decreased responsiveness of postsynaptic $GABA_A$ receptors we recorded action potential-independent, mIPSCs from the soma of BLA pyramidal-shaped neurons of KA-SE rats and sham control rats. mIPSCs were recorded at a holding potential of −70 mV, and in the presence of D-APV (50 μM), SCH50911 (20 μM), and GYKI 52466 (50 μM) and TTX (1 μM). The mean frequency of mIPSCs was 1.2 ± 0.4Hz (n = 14) in sham control rats and 0.5 ± 0.2 Hz in KA-SE rats (55.7 ± 3.9 % lower than the sham control group; n = 15, $p < 0.05$; Fig. 3). There was no significant difference in mIPSC amplitude, between sham control and KA-SE rats (Fig. 3). Thus, the reduced frequency and amplitude of sIPSCs (Fig. 2) did not involve a decreased responsiveness of postsynaptic $GABA_A$ receptors; the remaining possibilities are a significant interneuronal loss, reduced excitation of interneurons, and/or reduction in the probability of GABA release from GABAergic terminals.

Alterations in the GluR5 kainate receptor (GluR5KR)-mediated modulation of GABAergic transmission

An important mechanism regulating GABAergic synaptic transmission in the BLA operates via activation of GluR5KRs (Braga et al., 2003; Braga et al., 2004; Aroniadou-Anderjaska et al., 2007). The amygdala displays a high expression of the GluR5 subunit (Bettler et al., 1990; Li et al., 2001; Braga et al., 2003), and we have shown previously that GluR5KRs in the BLA are present on both postsynaptic (somatodendritic) and presynaptic sites of GABAergic interneurons. Activation of somatodendritic GluR5KRs enhances GABA release via depolarization of interneurons (Braga et al., 2003). Presynaptic GluR5KRs on GABAergic terminals increase the probability of GABA release when activated weakly, but have the opposite effect when the agonist concentration increases (Braga et al., 2003). Furthermore, there is evidence that GluR5KRs are significantly involved in epilepsy (Smolders et al., 2002; Gryder and Rogawski, 2003; Rogawski et al., 2003; Kaminski et al., 2004), and there are alterations in both the function of GluR5KRs (Palma et al., 2002) and the expression of the GluR5 subunit in MTLE patients (Mathern et al., 1998; Kortenbruck et al., 2001) and epileptic rats (Ullal et al., 2005). For these reasons, in the present study we examined if the reduced spontaneous inhibitory activity in the BLA (Fig. 2 and 3) was associated with alterations in the function of GluR5KRs or the expression level of the GluR5 subunit.

Bath application of the GluR5 agonist ATPA, at 300 nM, produced a 149 ± 31 % increase in the frequency of sIPSCs recorded from BLA pyramidal cells (n = 11) of sham control rats, while 1 μM ATPA further increased the sIPSC frequency to 419 ± 42 % from baseline (n = 11; Fig. 4). In the KA-SE rats, 300 nM ATPA produced only a 48 ± 22

% increase in sIPSC frequency from baseline (n = 10), and 1 μM ATPA increased sIPSC frequency to 218 ± 19 % from baseline (n = 10; Fig. 4). Thus, the enhancement in the frequency of sIPSCs in the BLA, produced by activation of GluR5KRs was significantly reduced in KA-SE rats compared to sham control rats, at agonist concentrations of either 300 nM ($p < 0.01$) or 1 μM ($p < 0.01$). As these effects of ATPA were probably produced by a depolarizing action on somatodendritic GluR5KRs of GABAergic interneurons, these results suggest that either the function of postsynaptic GluR5KRs present on GABAergic interneurons is impaired in the KA-SE rats, or the number of these receptors is reduced, which could be due to loss of GABAergic interneurons bearing GluR5KRs, reduced expression of the GluR5 subunit, or other mechanisms of downregulation.

When mIPSCs were recorded from BLA pyramidal cells, in the presence of TTX, 300 nM ATPA produced a 42.8 ± 7.2% facilitation in the mIPSC frequency (n = 9; Fig. 5A) in sham control rats, with no change in the amplitude or decay time constant of the mIPSCs. In contrast, in the KA-SE rats, 300 nM ATPA failed to produce a significant effect on mIPSCs (n = 9; Fig. 5A). Application of 1 μM ATPA produced a 31.6 ± 5.9 % reduction in the mIPSC frequency (n = 9; Fig. 5) in sham control rats, with no change in the amplitude or decay time constant of the mIPSCs, but had no significant effect on mIPSCs recorded from the KA-SE rats (n = 9; Fig. 5). These results suggest that presynaptic GluR5KRs in the KA-SE group are either downregulated or functionally impaired, or that there is a significant loss of GABAergic interneurons bearing presynaptic GluR5KRs.

Comparisons of the effects of ATPA in the BLA of slices from sham control rats and KA-SE rats in extracellular field potential recordings also indicated that GluR5KRs

were functionally impaired or their level was reduced in the KA-SE group. Thus, perfusion of 1 μM ATPA for 30 min reduced the amplitude of the field potential evoked in the BLA by stimulation of the external capsule to 80.0 ± 3.8 % of the baseline amplitude in sham control rat slices (n = 10), but did not cause a reduction in recordings from KA-SE rat slices (99.5 ± 3.5 % of the baseline amplitude; n = 12; Fig. 6). The reduction of the BLA field potentials by 1 μM ATPA in the sham control rats was reversible in all slices (not shown). The reduction may relate to the known effect of ATPA to enhance GABA release, which reduces the overall neuronal excitability and probability of action potential generation so that the population spike is reduced, or it may relate to presynaptic inhibition of glutamate release, which would also reduce excitability and action potential firing. Such reduction in the evoked field potentials by application of low concentrations of ATPA is also seen in the hippocampus (Vignes et al., 1998).

At 10 μM, ATPA induces epileptiform activity in the BLA (Rogawski et al., 2003; Aroniadou-Anderjaska et al., 2008), which could be due, at least in part, to presynaptic inhibition of GABA release (see the effect of 1 μM ATPA in Fig. 5 and 10 μM in Braga et al., 2003) and also to activation of GluR5KRs on BLA pyramidal cells (Gryder and Rogawski, 2003). When spontaneous activity was recorded in response to a 10 μM ATPA challenge, slices from sham control rats (n = 10) exhibited epileptiform bursting at higher frequency than did those from KA-SE rats (n = 12; Fig. 7), further supporting impairment of GluR5KR function.

Next, we used Western blot analysis to compare the expression level of the GluR5 protein in the BLA of KA-SE and sham control rats. We found that expression of the

GluR5 subunit in the BLA of KA-SE rats was reduced to 73.0 ± 4.6% of the level in sham control rats (Fig. 8; $p < 0.006$). For comparison, we also examined the GluR5 levels in two other brain regions. In the CA3 hippocampal area, there was no significant difference in the expression of the GluR5 subunit between sham control and KA-SE rats, while a significant elevation of the GluR5 protein was observed in the VPM/VPL thalamic nuclei of KA-SE rats (228.0 ± 24.4%; Fig. 8; $p < 0.02$).

Taken together, the results from electrophysiology and Western blot analysis indicate that the functional activity and expression of GluR5KRs is impaired in the BLA of KA-SE rats. Considering that GluR5KRs are activated by ambient concentrations of extracellular glutamate to facilitate GABA-mediated neurotransmission (Braga et al., 2003), the reduced activity of GluR5KRs in the KA-SE rats could contribute to the diminished frequency and amplitude of sIPSCs (Fig. 2) and the frequency of mIPSCs (Fig. 3) in non-ATPA stimulated slices from these animals.

Neuronal loss in the BLA

Our next step in investigating the alterations in the BLA of the KA-SE rats that may have contributed to the reduced inhibitory tone of pyramidal cells (Figs. 2 and 3) was to determine the extent of neuronal loss, and particularly the loss of GABAergic interneurons. To this end, we counted the total number of neurons and total number of GABAergic neurons in the BLA of the sham control and the KA-SE groups. The total number of neurons in the BLA of sham control rats (n = 5), estimated by stereologically counting Nissl-stained neurons, was 85,877 ± 2,913 (Table 1; Fig. 9). In the KA-SE rats (n = 5), the total number of neurons was 72,904 ± 1,261, a 15% reduction compared to

sham control rats ($p < 0.004$). The total number of GABAergic neurons in the BLA was estimated by counting GAD67-immunoreactive cells. GAD67 is the higher molecular weight (67 kD) isoform of glutamate decarboxylase (GAD), the enzyme that synthesizes GABA (Erlander and Tobin, 1991), and is predominantly localized in cell bodies of GABAergic neurons (Esclapez et al., 1994; Soghomonian and Martin, 1998). The total number of GABAergic neurons in the BLA of sham control rats was 11,685 ± 864 (Table 1, Fig. 9). In KA-SE rats, the total number of GABAergic neurons was 6,718 ± 537, a 43% reduction compared to controls ($p < 0.002$). Thus, in the BLA of sham control rats, GABAergic interneurons represent 13.7 ± 1.4% of the total population of neurons, but after KA-SE this is significantly reduced to 9.2 ± 0.7% (Fig. 9; $p < 0.02$), indicating that GABAergic neurons in the BLA are more vulnerable to KA-SE induced injury than principal cells.

Fluoro-Jade-C was used to determine the extent to which neurons are still degenerating in the BLA, 7–10 days after KA-SE. Whereas no Fluoro-Jade-C positive staining was found in the BLA of sham control rats ($n = 5$), all KA-SE rats ($n = 5$) demonstrated positive staining in the BLA (5.7 ± 2.1 FJ+ cells/section; Fig. 9; $p < 0.03$). In addition, positive staining was found, to a greater extent, in other amygdala nuclei, including the medial, lateral, posterior cortical, and central amygdala nuclei, as well as in other brain regions, such as the hilus, CA1, and CA3 subfields of the hippocampus, the piriform cortex, and the endopiriform cortex, in all KA-SE rats, but not in the control sham rats. These results demonstrate that neurodegeneration was still occurring in the BLA and other brain areas at 7 to 10 days after KA-induced status epilepticus.

$GABA_A$ α1 subunit expression

Our findings that the amplitude of mIPSCs is not altered in the BLA of the KA-SE rats (Fig. 3) suggest that the function and number of $GABA_A$ receptors on BLA pyramidal cells of the KA-SE rats was not altered post-status epilepticus. Other studies, however, have reported status epilepticus-induced alterations in the expression of different $GABA_A$ receptor subunits or $GABA_A$ receptor binding in different brain regions, including the amygdala (Gilby et al., 2005; Rocha et al., 2007), the hippocampus (Gibbs et al., 1997; Schwarzer et al., 1997; Tsunashima et al., 1997; Brooks-Kayal et al., 1998; Fritschy et al., 1999; Gilby et al., 2005; Raol et al., 2006), and thalamic nuclei (Rocha et al., 2007). To determine whether in the BLA of the KA-SE rats there were alterations in $GABA_A$ receptors that were not detectable with electrophysiological techniques, we measured the levels of the α1 $GABA_A$ receptor subunit ($GABA_A$ α1). $GABA_A$ α1 protein levels were significantly elevated in the BLA of the KA-SE rats to 257 ± 19% of the value in the sham control group (Fig. 8; $p < 0.003$). $GABA_A$ α1 levels were also increased in the CA3 subfield of the hippocampus (195 ±13% of shams; Fig. 8; $p < 0.003$), as well as in the VPM/VPL thalamic nuclei (157 ± 12% of shams; Fig. 8; $p < 0.04$) of the KA-SE rats. Given the results from the mIPSC recordings (Fig. 3), we have no evidence that the increased expression of $GABA_A$ α1 subunits influences the density or function of postsynaptic $GABA_A$ receptors on pyramidal neurons, at least at this stage of epileptogenesis.

Discussion

Kainic acid-induced status epilepticus has previously been shown to result in extensive neuronal damage in the amygdala that is particularly severe in the BLA (Pitkänen et al., 1998; Covolan and Mello, 2000), including a loss of GABA neurons (Tunnanen et al., 1996), but there is little information on the functional consequences of the neuronal loss on the activity of amygdala circuits. We now show that there is a profound reduction in GABA-mediated inhibitory activity in the BLA as assessed by recordings of spontaneous and miniature IPSCs. Our results confirm that there is damage to the BLA as demonstrated by an overall reduction in neuronal counts (15%), with a particularly dramatic loss of GABAergic neurons (43%), indicating that these neurons are selectively vulnerable. Previously, Sperk et al. (1983) observed that GAD enzyme activity was reduced 33% in amygdala/pyriform cortex of rats 9 days after kainate-induced status epilepticus, in good agreement with our estimates of the number of cells lost. We have also found that the remaining GABA neurons exhibit impaired ability to carry out GABA-mediated synaptic inhibition. This is manifest as defective modulation by GluR5KRs, a key mechanism regulating synaptic inhibition in the nucleus (Braga et al., 2004; Aroniadou-Anderjaska et al., 2007). Together, the drop-out of GABA neurons along with the impairment in the function of the surviving GABA neurons leads to reduced synaptic inhibition, which is likely to contribute to epileptic hyperexcitability manifest as epileptiform discharges and frank electrographic seizures (Fig. 1).

Reduced inhibitory tone in the BLA

The frequency and amplitude of action potential-dependent sIPSCs was reduced in BLA pyramidal neurons from KA-SE rats. In contrast, mIPSC amplitude was not reduced and the kinetic parameters were unaltered, although we did observe a reduction in mIPSC frequency. The pattern of effects is compatible with a loss of inhibitory GABA neurons and the defective function of GluR5KRs (as discussed in the next section). Although altered postsynaptic $GABA_A$ receptors have been observed in epileptic brain (McDonald et al., 1991; Henry et al., 1993; Rocha et al., 2007), the unaltered mIPSC amplitude and kinetics indicate that any changes in $GABA_A$ receptors were not functionally relevant, at least under our experimental conditions. In this regard, it is noteworthy that there are many reports of altered $GABA_A$ receptor subunit expression in brain regions including the amygdala (Gilby et al., 2005; Rocha et al., 2007) following status epilepticus. We also found an increase in $\alpha 1$ $GABA_A$ receptor subunit expression.

Impairments in GABA-mediated inhibitory transmission have been reported previously in the amygdala from epileptic animals (Gean et al., 1989; Smith and Dudek, 1997; Mangan et al., 2000; Benini and Avoli, 2006; for a review see Aroniadou-Anderjaska et al., 2008). In the BLA from rats with chronic limbic epilepsy (due to continuous hippocampal stimulation) characterized by spontaneous seizures and also in hippocampal kindled rats that had experienced thousands of seizures, Mangan et al. (2000) observed that spontaneous IPSPs in BLA neurons are absent. Our present results demonstrate that the inhibitory tone in the BLA network is reduced in the latent period, before the onset of spontaneous seizures.

The reduced inhibitory tone could contribute to epileptic hyperexcitability in the BLA and could be a factor in the occurrence of spontaneous seizures when the disease evolves from the latent phase to the epileptic chronic phase. As evidence that the BLA becomes hyperexcitable in association with the status epilepticus model, it has been show that kainate-treated epileptic rats manifest abnormal, repetitive population-spike responses to orthodromic stimulation (Smith and Dudek, 1997). This hyperexcitability may contribute to the seizure susceptibility of KA-SE rats. It is noteworthy that our recordings were carried out prior to the onset of overt spontaneous motor seizures. This raises the possibility that the early reduction of inhibitory activity could contribute to the process of epileptogenesis that leads eventually to spontaneous seizures, perhaps by permitting further excitotoxic damage to the amygdala (Nairismagi et al., 2004). In fact, we did observe ongoing neuronal degeneration, although the cause is not known.

Impaired GluR5KR-mediated modulation of GABA release

Unlike other ionotropic glutamate receptor subunits, GluR5KR subunits have a relatively restricted distribution in the central nervous system, but are highly expressed in the BLA (Bettler et al., 1990; Li et al., 2001; Braga et al., 2003). We have previously shown that postsynaptic GluR5KRs on somatodendritic sites of BLA interneurons enhance GABA release by depolarizing interneurons. Presynaptic GluR5KRs on GABAergic terminals also facilitate GABA release when activated by low concentrations of an agonist, but inhibit GABA release when activated by higher agonist concentrations (≥1 μM ATPA; Braga et al., 2003). GluR5KRs are also present on principal neurons where they have a depolarizing action (Gryder and Rogawski, 2003); it is not known whether there are

presynaptic GluR5KRs on these neurons that affect glutamate release. The net effect of GluR5KR activation on the BLA network is to reduce excitability when agonist concentrations are low, while epileptiform activity is induced with high agonist concentrations due to direct excitation of principal neurons and reduced GABA release from interneurons. In the present study, the reduced enhancement of sIPSCs by ATPA in the KA-SE group, and the near absence of an ATPA effect on mIPSCs indicates that there is impaired function of GluR5KRs on surviving GABA neurons or, alternatively, that GluR5KR-bearing GABA neurons are preferentially lost. Additional evidence of defective GluR5KR receptor function comes from recordings of field potentials where, under ordinary condition, 1 μM ATPA causes a reduction in the field potential amplitude; this action was absent in the KA-SE group. Finally, 10 μM ATPA caused a reduced frequency of epileptiform bursting in the BLA in slices from KA-SE rats, which also may reflect impaired GluR5KR functional activity.

Loss of GABAergic interneurons

In agreement with prior studies (Tuunanen et al., 1996, 1999), we observed a substantial reduction in the number of neurons in the BLA after kainate-induced status epilepticus. However, the magnitude of the reduction was less than in these previous studies most likely because the duration of status epilepticus in our experiments was shorter. It is well recognized that the severity of neuronal damage in status epilepticus models is dependent on the duration of seizure activity (Lemos and Cavalheiro, 1995; Gorter et al., 2003). In the present study, we used diazepam to terminate seizure activity within 3 hours after onset whereas Tuunanen et al. (1996) recorded seizure activity lasting on average 13

hours. As observed in animal models, it is interesting to note that loss of neurons in the BLA is also a common pathological feature in human MTLE (Cendes et al., 1993; Hudson et al., 1993; Wolf et al., 1997; Pitkänen et al., 1998; Guerreiro et al., 1999).

The damage to GABA neurons in the present study was significantly greater than that of principal neurons, suggesting a higher vulnerability of GABA neurons to status epilepticus-induced injury. It has been speculated that certain interneuron populations are vulnerable to seizure-induced damage because of a low capacity to buffer calcium by calcium binding proteins (Sloviter, 1989; Scharfman and Schwartzkroin, 1989); however no clear relationship has been established between calcium binding proteins and vulnerability to damage by prolonged seizures (Freund et al., 1992). A high susceptibility of somatostatin-containing interneurons to status epilepticus-induced damage has been reported in the hippocampus (Sloviter, 1987; Buckmaster and Dudek, 1997; Sun et al., 2007), and has been associated with the high level of a tyrosine phosphatase present in these neurons, which blocks a latent neuroprotective response initiated by the ERK/MAPK signaling pathway (Choi et al., 2007). A similar mechanism could account for the vulnerability of interneurons in the BLA (Tuunanen et al., 1996; 1997). However, since only 11–18% of BLA GABAergic neurons contain somatostatin (McDonald and Pearson, 1989), other types of GABA neurons are probably also affected.

Functional Implications

In hyperexcitable, epileptic neuronal circuits, regardless of the underlying mechanisms that have led to hyperexcitability, the characteristic end result is a derangement in the balance between excitatory and inhibitory activity. Although alterations in excitatory

glutamatergic transmission can underlie this imbalance, impaired GABA-mediated inhibition is most often also involved (Coulter, 1999; El-Hassar et al., 2007). In the post-status epilepticus BLA, there is clearly impairment of GABAergic inhibition. It remains to be determined whether there are also changes in excitatory synaptic function in this brain structure.

Alterations in GluR5KRs play a key role in the impairment of GABAergic inhibition. When extracellular glutamate levels rise, as during epileptic activity, GluR5KRs contribute to hyperexcitability by depolarizing principal neurons and depressing interneuron-mediated GABAergic inhibition. Accordingly, studies in brain slices and with GluR5 knockout mice have confirmed that GluR5KR activation can trigger epileptiform discharges and seizures, and GluR5 antagonists block epileptiform activity in amygdala slices (Rogawski et al., 2003; Fritsch et al., 2006; Apland et al., 2007) as well as limbic seizures in vivo (Smolders et al., 2002). In the post-status-epilepticus BLA, since GABAergic inhibition is already markedly impaired due to a selective loss of GABA neurons, defective GluR5 function is not expected to protect against seizures, especially since excitation can be expressed through other ionotropic glutamate receptors. However, under ordinary conditions when the BLA network is relatively quiet and extracellular levels of glutamate are low, GluR5KRs contribute to maintaining GABA-mediated inhibitory tone by facilitating GABA release from interneurons via both postsynaptic and presynaptic mechanisms (Braga et al., 2003; Aroniadou-Anderjaska et al., 2007). Therefore, hyperexcitability in the post-status-epilepticus BLA may be promoted by a reduction in this inhibitory tone due to impaired GluR5KR mechanisms. During the latent period, this hyperexcitability is expressed as

epileptiform activity, but not motor seizures. However, in the subsequent chronic period, seizure discharges in the BLA, which can spread to other limbic structures via its extensive projections (Amaral et al., 1992; Pitkänen, 2000), may contribute to the propensity for spontaneous behavioral seizures.

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Table 1. Stereological estimation of total Nissl-stained and GAD67-positive neurons in the BLA of sham control rats and KA-SE rats

	Sham Control (n = 5)	KA-SE (n = 5)	*p* value (*t* test)	% Neurons Remaining
Nissl-stained				
Mean	85,877	72,904	<.004	84.9
SEM	2,913	1,261		
Mean Gundersen CE (m=1)	.060	.062		
Mean Schmitz-Hof CE (2nd est)	.056	.061		
GAD67+				
Mean	11,685	6,718	<.002	57.4
SEM	864	537		
Mean Gundersen CE (m = 1)	.066	.076		
Mean Schmitz-Hof CE (2nd est)	.065	.076		

Means are bilateral values; n indicates number or rats per group. SEM, standard error of the mean; CE, coefficient of error as calculated by Gundersen et al. (1999) and Schmitz and Hof (2000). Glutamate decarobxylase (GAD67) positive neurons were labeled using GAD67 immunohistochemistry.

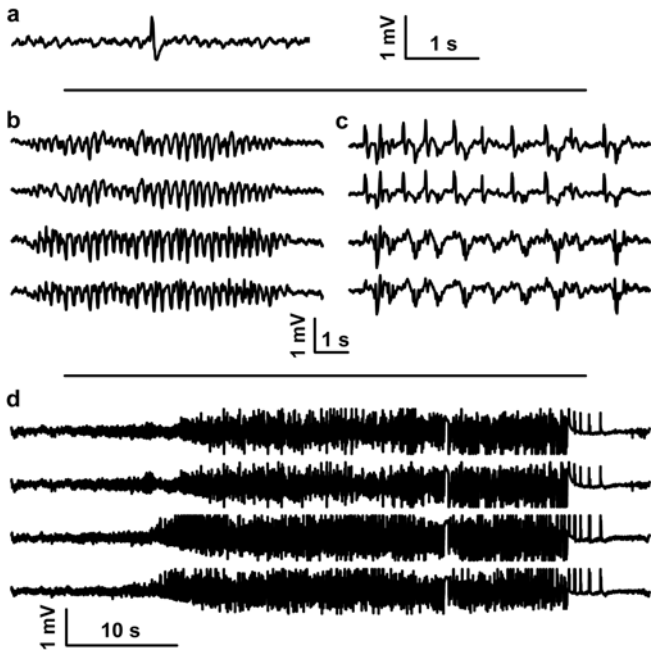

Figure 1. EEG traces recorded from the cortex of rats 6 days after KA-SE. (**a**) single sharp wave, (**b**) spike-wave complexes, (**c**) periodic generalized epileptic discharges, and (**d**) seizures. Trace in (**a**) was recorded from a left frontal cortical screw electrode. Traces in (**b**), (**c**), and (**d**) were recorded (top to bottom) from a left frontal, right frontal, left parietal, and right parietal cortical screw electrode.

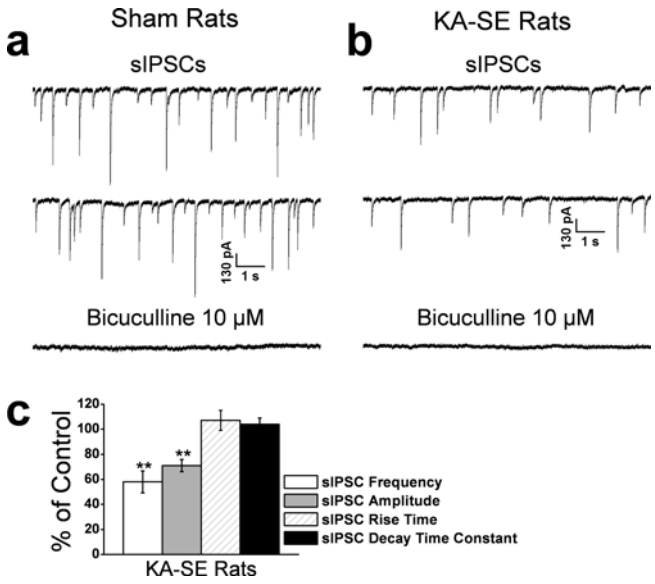

Figure 2. The frequency and amplitude of spontaneous IPSCs (sIPSCs) in BLA neurons are reduced on days 7 to 10 after KA-SE. sIPSCs were recorded from pyramidal-shaped neurons in the presence of D-APV (50 μM), SCH50911 (20 μM), and GYKI 52466 (50 μM), at a holding potential of −70 mV. The frequency and the amplitude of the sIPSCs were reduced in a neuron from a KA-SE rat (**b**) compared to one from a sham control (**a**). The recorded currents in both cases were blocked by the $GABA_A$ receptor antagonist bicuculline. **(c)** Group data from 21 slice recordings from KA-SE rats, normalized to the values obtained from sham controls. The frequency and amplitude but not the rise time and the decay time constant of the sIPSCs were significantly reduced in neurons from the KA-SE rats compared to those from the sham controls (**, $p < 0.01$).

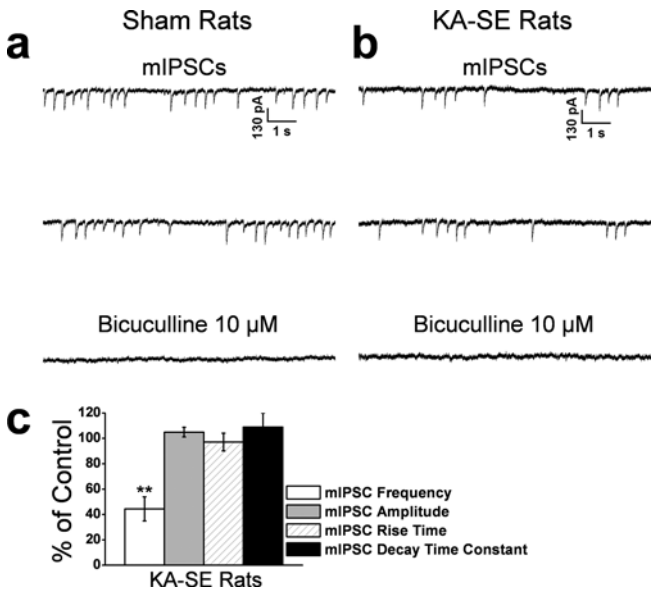

Figure 3. The frequency but not the amplitude of miniature IPSCs (mIPSCs) in BLA neurons is reduced on days 7 to 10 after KA-SE. mIPSCs were recorded in the presence of D-APV (50 μM), SCH50911 (20 μM), GYKI 52466 (50 μM), and TTX (1 μM), at a holding potential of −70 mV. The frequency of the mIPSCs was reduced in a neuron from a KA-SE rat **(b)** compared to one from a sham control **(a)**. The recorded currents in both cases were blocked by the $GABA_A$ receptor antagonist bicuculline. **(c)** Group data from 15 slice recordings from KA-SE rats, normalized to the values from sham controls. The frequency, but not the amplitude, rise time, or decay time constant of the mIPSCs was significantly reduced in neurons from the KA-SE rats compared to those from the sham controls (**, $p < 0.01$).

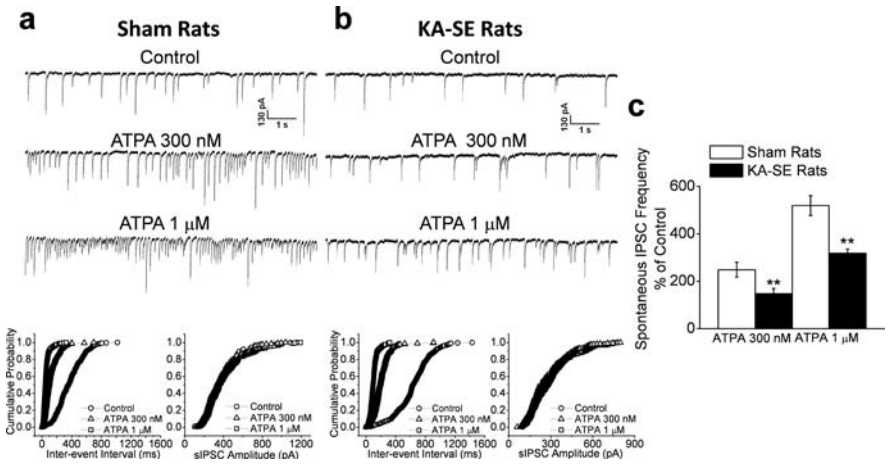

Figure 4. The GluR5KR-mediated enhancement in the frequency of sIPSCs in the BLA is reduced after KA-SE. The GluR5KR agonist ATPA, at 300 nM or 1 μM, increased the frequency of sIPSCs recorded from BLA pyramidal neurons in sham control rats **(a)**, but its effect was blunted in pyramidal neurons from KA-SE rats **(b)**. The cumulative probability plots of interevent intervals and amplitudes of sIPSCs corresponding to the traces in **(a)** and **(b)** are shown at the bottom. **(c)** Pooled sIPSC frequency-data of 10 slices from KA-SE rats and 11 slices from sham rats, expressed as a percentage of the sIPSC frequency during control conditions (before application of ATPA). The increase in sIPSC frequency by either concentration of ATPA was significantly lower in the KA-SE rats compared to the sham rats (**, $p < 0.01$).

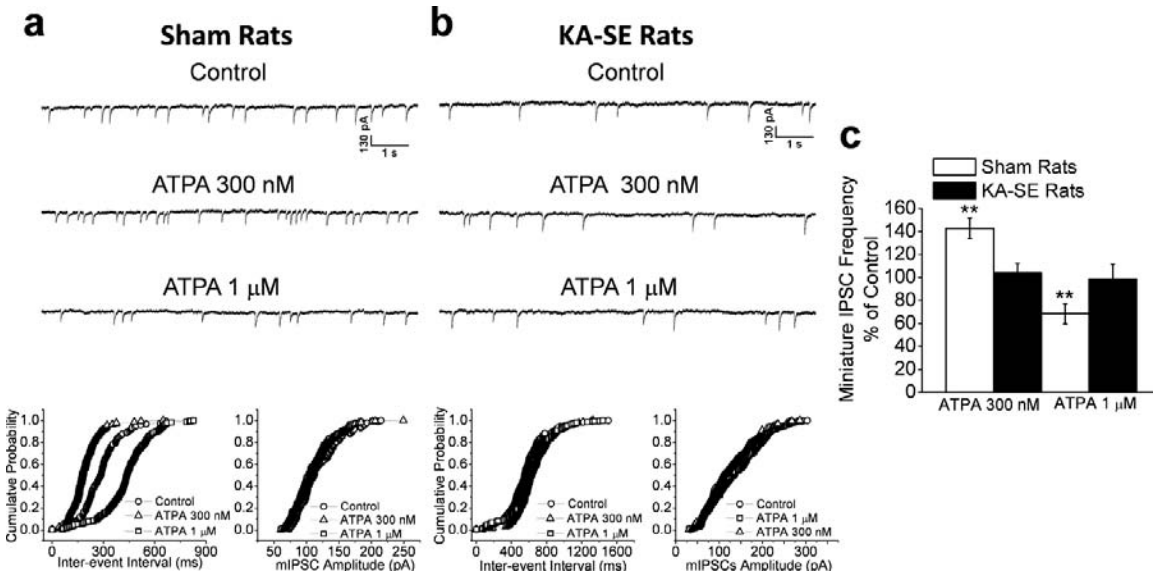

Figure 5. GluR5KR-mediated effects on presynaptic GABA release as assessed by recordings of mIPSCs are nearly absent in BLA pyramidal neurons from KA-SE rats. In pyramidal neurons from sham control rats, the GluR5KR agonist ATPA, at 300 nM, increased the frequency of mIPSCs **(a**); this effect was almost absent in neurons from KA-SE rats **(b)**. At 1 μM, ATPA reduced the frequency of mIPSCs in neurons from the sham group **(a**), but had virtually no effect on neurons from the KA-SE group **(b**). The cumulative probability plots of interevent intervals and amplitudes of mIPSCs corresponding to the traces in **(a)** and **(b)** are shown at the bottom. **(c)** Pooled mIPSC frequency-data from 9 KA-SE rat slices and 9 sham control rat slices expressed as a percentage of the mIPSC frequency before application of ATPA (**, $p < 0.01$).

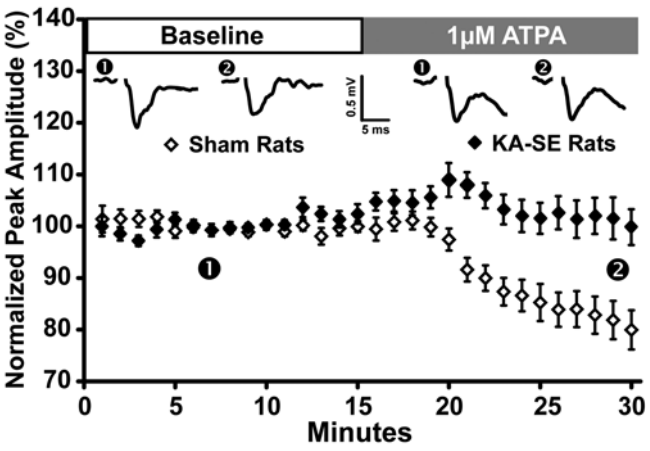

Figure 6. Activation of GluR5KRs by 1 μM ATPA reduces the BLA field potential in slices from sham control rats but not in slices from KA-SE rats. Field potentials in the BLA were evoked by stimulation of the external capsule. ATPA (1 μM) reduced the amplitude of the field potentials in slices from the sham rats, but had no significant effect in those from the KA-SE rats. Sample traces from a sham rat slice, before and in the presence of ATPA, are shown to the left, and traces from a KA-SE rat slice are shown to the right. Numbers indicate the point in the time course of the sample traces. The data points indicate the mean ± SEM of peak amplitude values from 12 KA-SE rat slices and 10 sham control rat slices.

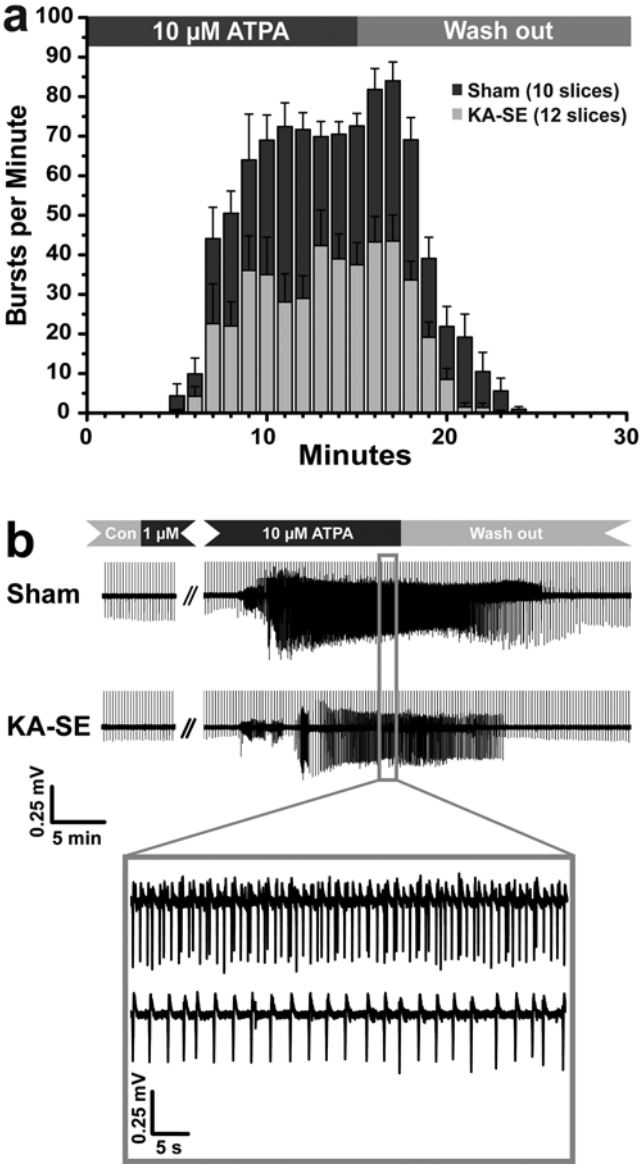

Figure 7. ATPA (10 µM) induces lower frequency spontaneous epileptiform bursting in BLA slices of KA-SE rats than in those from sham rats. (**a**) Frequency of bursts in the presence of ATPA and during wash-out, for the sham and the KA-SE groups. The frequency of bursts was significantly higher in the slices from sham rats at all time points ($p < 0.05$). (**b**) Representative examples of the effects of 10 µM ATPA on spontaneous activity in BLA slices from sham and KA-SE rats.

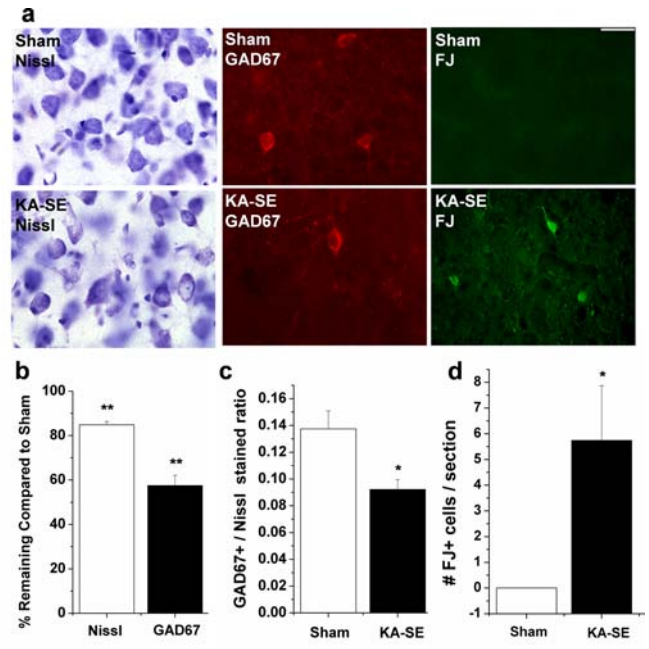

Figure 8. GABAergic interneurons are preferentially lost in the BLA after KA-SE, and neurodegeneration continues on days 7 to 10 after KA-SE**. (a)** Photomicrographs of Nissl-stained, GAD67-immunoreactive, and Fluoro-Jade (FJ)-stained sections from the BLA of sham rats and KA-SE rats 7–10 days after KA-SE. **(b)** Number of Nissl-stained neurons and GAD67-immunoreactive neurons in the KA-SE rats (n = 5), expressed as a percentage of the number of neurons in the sham-control group. **(c)** Ratio of GAD67-immunoreactive neurons to Nissl-stained neurons in the BLA of sham control and KA-SE rats, demonstrating that a reduced number of GABA neurons make up the total population of neurons in the BLA of the KA-SE rats compared to sham control rats. Mean ± SEM values from 5 sham rats and 5 KA-SE rats. **(d)** Numbers of Fluoro-Jade-positive cells in the BLA of KA-SE rats (n = 5) and sham control rats (n = 5). Fluoro-Jade positive cells were present in the BLA of the KA-SE rats indicating ongoing neurodegeneration; no Fluro-Jade positive cells were noted in the BLA of sham control rats. *, $p < 0.05$; **, $p < 0.005$. Scale bar, 25 μm.

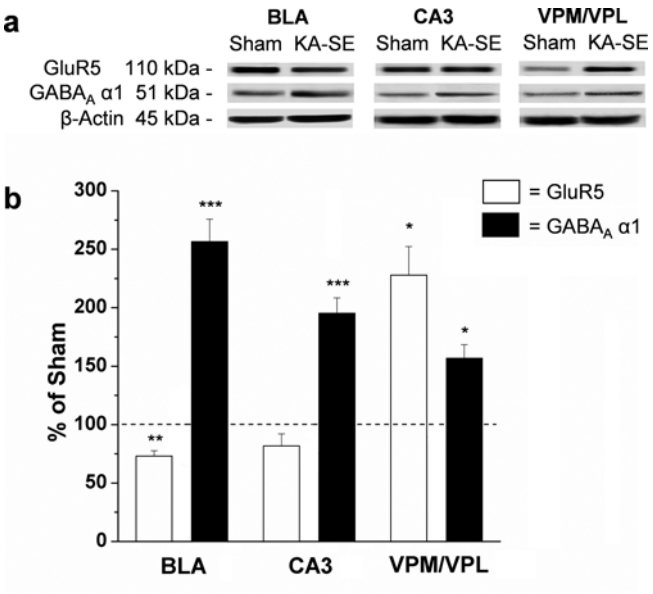

Figure 9. GluR5 kainate receptor and $GABA_A$ receptor α1 subunit protein expression in amygdala, hippocampus and thalamus of KA-SE rats. **(a)** Representative Western blots showing GluR5, $GABA_A$ α1, and β-actin antibody binding, in the BLA, CA3 subfield of the hippocampus, and the ventral posteromedial and ventral posterolateral thalamic nuclei (VPM/VPL). **(b)** Quantification of GluR5 and $GABA_A$ α1 protein densities relative to β-actin density in KA-SE rats, expressed as a percent of the value in sham control rats. Values are mean ± SEM (n = 6); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

CHAPTER 3

Diazepam administration after prolonged status epilepticus reduces neurodegeneration in the amygdala but not in the hippocampus during epileptogenesis.

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Key words: amygdala, hippocampus, benzodiazepines, status epilepticus, neurodegeneration, epileptogenesis

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Abstract

An episode of status epilepticus (SE), if left untreated, can lead to death, or brain damage with long-term neurological consequences, including the development of epilepsy. The most common first-line treatment of SE is administration of benzodiazepines (BZs). However, the efficacy of BZs in terminating seizures is reduced with time after the onset of SE; this is accompanied by a reduced efficacy in protecting the hippocampus against neuronal damage, and is associated with impaired function and internalization of hippocampal $GABA_A$ receptors. In the present study, using Fluoro Jade-C staining, we found that administration of diazepam to rats at 3 hours after the onset of kainic acid-induced SE, at a dose sufficient to terminate SE, had no protective effect on the hippocampus, but produced a significant reduction in neuronal degeneration in the amygdala, piriform cortex, and endopiriform nucleus, examined on days 7 to 9 after SE. Thus, in contrast to the hippocampus, the amygdala and other limbic structures are responsive to neuroprotection by benzodiazepines after prolonged SE, suggesting that $GABA_A$ receptors are not significantly altered in these structures during SE.

Introduction

Status epilepticus (SE) in humans is an acute medical emergency associated with significant morbidity and mortality (Krumholz et al. 1995; Cascino et al. 1998; Logroscino et al. 2002). Seizures during SE are generalized and enduring, and if not controlled timely, brain damage may occur (Treiman 2007) leading to neurological, cognitive, or other behavioral deficits (Krumholz et al. 1995). Furthermore, after an episode of SE, patients have a high risk of developing epilepsy (Hesdorffer et al. 1998). This is because SE can trigger epileptogenesis, a process whereby –in the absence of further seizures after the initial SE episode– structural and functional alterations take place in certain brain regions that play a key role in epilepsy, culminating in the appearance of spontaneous seizures and eventually the development of epilepsy. SE has been successfully used in animal models as a trigger for epileptogenesis and the development of temporal lobe epilepsy (TLE). In the SE animal model, neuronal loss and reorganization of neuronal circuits during epileptogenesis are similar to neuropathological findings in TLE patients (for a review see Pitkänen et al. 2007), particularly in regard to the neurodegeneration that occurs in limbic structures (Tuunanen et al. 1996; Covolan and Mello 2000; Aroniadou-Anderjaska et al. 2008).

The first-line treatment for SE is administration of benzodiazepines (BZs), which increase the efficacy of $GABA_A$ receptor-mediated inhibition by binding to specific sites on $GABA_A$ receptors and allosterically modulating the $GABA_A$ receptor complex (Macdonald and Olsen 1994; McKernan and Whiting 1996). However, BZs lose their efficacy if not administered early (Treiman et al. 1998), and higher concentrations, or more aggressive second- and third-line antiepileptic drugs must be used (Bleck 1999;

Jones et al. 2002; Riss et al. 2008). The weakening anticonvulsant effect of BZs with increasing duration of SE has been associated with impaired function and internalization of $GABA_A$ receptors in the hippocampus (Goodkin et al. 2005; Naylor et al. 2005; Feng et al. 2008). This is consistent with the lack of protection against neuronal loss in the hippocampus when the BZ diazepam is administered 3 hours after the onset of SE (Pitkänen et al. 2005). It is unknown, however, if other limbic structures that play an important role in seizure generation also lose their responsiveness to BZs soon after the onset of SE. Despite the loss of hippocampal responsiveness to BZs as SE progresses, these drugs maintain some effectiveness in reducing seizures, particularly if the doses are increased (Treiman. 1990; Jones et al. 2002). This suggests that other brain regions that are important in sustaining status epilepticus may remain responsive to benzodiazepines even at later stages of SE. As a first step in investigating this question, we compared the protective effect of diazepam against neuronal degeneration in the hippocampus and in the amygdala, as well as the piriform cortex and endopiriform nucleus, when the drug is administered at 3 hours after SE. Neuronal degeneration was examined on days 7 to 9 after SE.

We studied the amygdala because of its important role in TLE (Quesney 1986; Pitkänen et al. 1998; Aroniadou-Anderjaska et al. 2008), as well as in the generation and spread of seizure activity in the SE and kindling animal models of TLE (White and Price 1993a,b; Mohapel et al. 1996). The volume of the amygdala is often reduced in TLE patients (Cendes et al. 1993; Cendes et al. 1994; Wolf et al. 1997; Van Paesschen et al. 2001), while in SE animal models the amygdala suffers extensive neuronal loss (Tuunanen et al. 1996; Hsieh 1999). Similarly, the piriform cortex and the adjacent

endopiriform nucleus also undergo substantial neuronal loss after SE (Ben-Ari, 1985; Covolan and Mello 2000; Druga et al. 2003; Chen and Buckmaster 2005) and may play an important role in epileptogenesis (for a review see Majak and Moryś, 2007). We found that diazepam did not reduce neuronal degeneration in the hippocampus, while it had a significant protective effect in the amygdala, the piriform cortex and the endopiriform nucleus.

Materials and Methods

Animals. Experiments were performed on male Sprague Dawley rats (Taconic Farms, Rockville, MD), 5-6 weeks old, weighing 170-200 g at the start of the experiments. Animals were individually housed in an environmentally controlled room (20–23°C, 12-h light/12-h dark cycle, lights on 06:00 am), with food and water available *ad libitum*. All animal experiments were in accordance with our institutional guidelines after obtaining approval of the Institutional Animal Care and Use Committee (IACUC).

Induction of Status Epilepticus. Five rats implanted with four cortical screw electrodes plus 4 non-implanted rats were injected (i.p.) with kainic acid (KA) to induce SE, following a modified titration protocol (Hellier et al. 1998). The electrode-implanted rats were allowed one week of recovery before KA injections. Rats were initially treated with 7.5-8 mg/kg KA dissolved in 0.1 M phosphate buffered normal saline (PBS; 5mg/ml), followed by subsequent doses of 4-5 mg/kg until the onset of SE. For implanted rats, SE onset was defined by 5 min of continuous generalized electrographic seizure activity in all four cortical electrodes involved with no breaks longer than 10 seconds; SE was

allowed to continue for 180 minutes, until termination of both behavioral and electrographic seizures with 25 mg/kg of diazepam (i.p.). This group of rats will be referred to as KA+DZP group (n = 5). The four non-implanted rats were given KA to induce SE, using behavioral assessments to determine the necessity of additional injections and the onset of SE (defined by the first generalized behavioral seizure; Stage 4 from Racine 1972). In this group, diazepam was not used to terminate SE (KA group). Behavioral SE was observed for more than 4 hours after the first Stage 4 behavioral seizure. SE was still ongoing when monitoring was stopped. There were no mortalities. Previous studies have shown that SE induced by KA results in behavioral seizures that can continue for more than 7 hours (Tuunanen et al. 1999), and epileptiform spiking for up to 12-14 hours (Pitkänen et al. 2005) after KA injections. The KA group received 16.6 ± 0.67 KA injections, and the KA +DZP group received 20.5 ± 2.55 KA injections to induce SE (no significant difference between the number of KA injections needed in the two groups; $P < 0.31$).

The KA +DZP group was electrode-implanted so that we could determine the efficacy of diazepam to terminate electrographic seizures in addition to the behavioral seizures. In previous experiments, we have examined neuronal degeneration (using Fluoro-Jade C) in "sham rats" that were electrode-implanted but received no other treatment, and control rats which did not receive any treatment. There were no degenerating cells in the amygdala or hippocampus in either group. Therefore, the electrode implantation in the group which received diazepam is unlikely to have affected the results.

Fixation & Tissue Processing. The four rats of the KA group and the five rats of the KA+DZP group were used for morphological analysis of the amygdala, hippocampus, piriform cortex, and endopiriform nucleus. Seven to nine days after KA-induced SE , rats were deeply anesthetized using ketamine (60 mg/kg i.p.) and medetomidine (0.5 mg/kg i.p.) and transcardially perfused with PBS (100 mL) followed by 4% paraformaldehyde (250 mL). The brains were removed and post-fixed overnight at 4º C, then transferred to a solution of 30% sucrose in PBS for 72 hours, and frozen with dry ice before storage at -80º C until sectioning. A 1-in-6 series of sections containing the rostro-caudal extent of the amygdala was cut at 40 µm on a sliding microtome. One series of sections was mounted on slides (Superfrost Plus, Daigger, Vernon Hills, IL) in PBS for Nissl staining with cresyl violet. An adjacent series of sections was also mounted on slides for Fluoro-Jade C (FJ) staining.

Fluoro-Jade C Staining. Fluoro-Jade C (Histo-Chem, Jefferson, AK) was used to identify dying neurons in the amygdala, hippocampus, piriform cortex, and endopiriform nucleus, at 7-9 days after SE. Mounted sections were air-dried overnight, and then immersed in a solution of 1% sodium hydroxide in 80% ethanol for 5 min. The slides were then rinsed for 2 min in 70% ethanol, 2 min in dH_20, and incubated in 0.06% potassium permanganate solution for 10 min. After a 2 min rinse in dH_20, the slides were transferred to a 0.0001% solution of Fluoro-Jade C dissolved in 0.1% acetic acid for 10 minutes. Following three 1-minute rinses in dH_20, the slides were dried on a slide warmer, cleared in xylene for at least 1 min and coverslipped with DPX (Sigma).

Evaluation of Fluoro-Jade C. Tracings of the amygdala, dorsal hippocampus, piriform cortex, and endopiriform nucleus from an adjacent series of Nissl-stained sections were superimposed on the FJ-stained sections. For qualitative analysis of FJ-stained sections, the following rating system was used to determine the score for the extent of degeneration in each structure: 0 = no damage; 1 = minimal damage (1-10%); 2 = mild damage (10-25%); 3 = moderate damage (25-45%); and 4 = severe damage (>45%). Qualitative assessments were made from 6 sections per animal, and the average for each animal was recorded. For quantitative analysis, FJ positive cells were counted in each outlined structure at 20×, and recorded as a density (number of cells per mm^2) from, on average, 6 sections.

Statistical Analysis All statistical values are presented as mean ± SEM. The KA group and the KA +DZP group were compared using the unpaired Student's *t* test. Differences between the two groups were considered statistically significant when $P < 0.05$. Sample sizes (n) refer to the number of rats.

Results

Neuropathology of Limbic Structures

Neuronal damage of limbic structures occurs both acutely (Tuunanen et al. 1999; Covolan and Mello 2000) and as a delayed process in the weeks, or even months, after SE (Tuunanen et al. 1996; Chen and Buckmaster 2005). In the present study, degenerating neurons were identified using FJ, a fluorescent marker that binds to irreversibly damaged neurons (Schmued et al. 1997). We characterized the extent of

neurodegeneration in different limbic structures during epileptogenesis triggered by KA-induced SE in two groups: rats in which SE was not terminated with diazepam (KA rats; n = 4) and rats in which SE was terminated after 3 hours with diazepam (KA +DZP rats; n = 5). In both groups, the region with the most extensive ongoing degeneration, having the highest density of FJ+ cells, was the endopiriform nucleus, followed by the amygdala (Table 1; Figure 3). To a lesser extent, neurodegeneration was also observed in the piriform cortex and hippocampus (Table 1; Figure 3).

Hippocampus

In the hippocampus of the KA rats, 18 ± 5 FJ+ cells were counted (see methods), on days 7 to 9 after KA-induced SE. The extent of neurodegeneration was not significantly different from that in the KA +DZP rats, in which 25 ± 12 FJ+ cells were counted in the hippocampus (Figures 1 and 3; Table 1). The qualitative scoring system also showed no significant difference in these groups, with a score of 1.71 ± 0.29 for the KA group and 1.50 ±0.46 for the KA +DZP group. The most extensively damaged subfields were the CA3, CA1 and hilar region. Thus, administration of diazepam at 3 hours after the onset of SE has no effect on the extent of neurodegeneration in the hippocampus, 7 to 9 days after SE.

Amygdala

The amygdala plays a central role in the generation and spread of seizure activity in animal models of epileptogenesis (White and Price 1993a,b; Mohapel et al. 1996). Benzodiazepine-sensitive $GABA_A$ receptors are present in the amygdala (Sieghart and

Sperk 2002). In the present study, we found extensive neurodegeneration in the amygdala, 7-9 days after SE, which was significantly reduced by diazepam. The qualitative analysis revealed a score of 2.88 ± 0.50 for the KA group, which was significantly higher than the score of 1.23 ± 0.34 in the KA +DZP group ($p < 0.05$). In the quantitative analysis, we counted 105 ± 5 FJ+ cells/mm^2 in the amygdala of the KA rats. This number was significantly reduced (by 63%) in the KA +DZP group, in which 39 ± 11 FJ+ cells were counted (Figures 2 and 3; Table 1; $P < 0.005$). Thus, diazepam administered after prolonged SE reduces neuronal degeneration in the amygdala, 7 to 9 days after SE.

Piriform Cortex & Endopiriform Nucleus

The piriform cortex and the adjacent endopiriform nucleus are also susceptible to damage after SE (Ben-Ari 1985; Covolan and Mello 2000; Druga et al. 2003; Chen and Buckmaster 2005). In the present study, we determined if terminating SE after 3 hours with diazepam reduces neurodegeneration in these two brain regions. At 7-9 days after SE, the qualitative evaluation score for neurodegeneration in the piriform cortex was 3.63 ± 0.10 for the KA group, compared to 1.30 ± 0.37 for the KA +DZP group, indicating that diazepam significantly ($P < 0.001$) reduced the extent of neurodegeneration (Figure 3; Table 1). Similarly, we found a significant reduction (77%; $P < 0.05$) in the number of degenerating cells in the piriform cortex of the KA +DZP rats (Figures 2 and 3; Table 1). Thus, in the KA group we counted 42 ± 10 FJ+ cells, while the number of FJ+ cells in the KA +DZP group was 10 ± 3.

The endopiriform nucleus had the densest FJ+ cells compared to the other structures (Figures 2 and 3; Table 1). The qualitative evaluation score for neurodegenerating cells in the endopiriform nucleus of rats in the KA group was 3.50 ± 0.40, whereas in the KA +DZP group it was significantly lower (1.77 ± 0.28; $P < 0.01$). Similarly, the number of FJ+ cells in the KA group was 194 ± 22, while the number of FJ+ cells in the KA +DZP group was 102 ± 28, a 47% reduction ($P < 0.05$; Figures 2 and 3, Table 1).

Discussion

In the present study, we found that neurodegeneration occurring at 7-9 days after KA-induced SE is more extensive in the amygdala and the endopiriform nucleus compared to the piriform cortex and the hippocampus, and that SE termination after 3 hours by administration of diazepam results in significant protection from neurodegeneration in the amygdala, endopiriform nucleus, and piriform cortex, but not in the hippocampus.

Atrophy of temporal lobe structures, which implies primarily neuronal loss, is a frequently reported histopathology of TLE patients (Babb and Brown 1989; Babb 1991; Cendes et al. 1993; Hudson et al. 1993; Williamson et al. 1993; Cendes et al. 1994). Similarly, in animal models of TLE, neuronal damage of limbic structures is evident after an epileptogenic insult (Schwob et al.1980; Heggli and Malthe-Sørensson 1982; Tuunanen et al. 1996; Tuunanen et al. 1999; Covolan and Mello 2000; Chen and Buckmaster 2005). The amygdala appears to be particularly vulnerable to seizure-induced neuronal damage, as seen either acutely after SE (Schwob et al. 1980; Tuunanen

et al. 1999, Covolan and Mello 2000), during the epileptogenic latent period (Heggli and Malthe-Sørensson 1982; Tuunanen et al. 1996, Qashu et al. 2008), or after spontaneous seizures have developed (Chen and Buckmaster 2005). By using a marker specific for *irreversibly* degenerating cells (Schmued et al. 1997), we show here that the extent of ongoing neurodegeneration during epileptogenesis is greater in the amygdala and the endopiriform nucleus compared to the hippocampus and piriform cortex. A higher susceptibility of the amygdala to seizure-induced brain damage compared to the hippocampus has also been observed in previous studies after KA-induced SE (Schwob et al. 1980; Heggli and Malthe-Sørensson 1982; Riba-Bosch and Perez-Clausell 2004; Chen and Buckmaster 2005), but also after SE induced by other mechanisms, such as administration of nerve agents (Shih et al. 2003). There is evidence suggesting that the amygdala is even more prone to generating seizure activity than the hippocampus (Goddard 1967; Kairiss et al. 1984; Racine et al. 1988), and, therefore, during SE the amygdala perhaps suffers more intense seizures than the hippocampus, resulting in greater neuropathological damage. It is not clear at present if there are also other mechanisms related to the physiology and biochemistry of the amygdala that make this structure more vulnerable to seizure-induced neuronal damage than other brain regions, even when the intensity and duration of the seizures are not higher in the amygdala. The endopiriform nucleus is another brain region that is highly susceptible to seizure-induced neuronal damage, as revealed in the present study, as well as in previous studies (Covolan and Mello 2000; Druga et al. 2003; Chen and Buckmaster 2005).

GABAergic interneurons in the amygdala are the most vulnerable to SE-induced damage (Tuunanen et al. 1996). We have recently found that in the basolateral

amygdala, the amygdala nucleus that plays the most central role in the initiation and propagation of seizure activity (White and Price 1993a,b; Mohapel et al. 1996), the loss of GABAergic neurons is significantly greater than the loss of other cells, 7-10 days after SE, resulting in a dramatic reduction of inhibitory activity (Qashu et al. 2008). Thus, because it is GABAergic neurons that are primarily lost in the amygdala after SE, the amygdala circuitry becomes hyperexcitable, which may contribute significantly to the progression of epileptogenesis and the development of epilepsy. It is important therefore to protect against SE-induced neuronal loss in the amygdala.

We found in the present study that diazepam administration to terminate SE reduced neuronal degeneration in the amygdala, endopiriform cortex and piriform cortex, but not in the hippocampus. Benzodiazepine (BZ)-sensitive receptors are densely located in limbic structures (Niehoff and Kuhar 1983; Fritschy and Mohler 1995), and BZs are the first-line treatment for termination of SE in humans (Chen and Wasterlain 2006), and reduction of mortality after prolonged SE in animal models of TLE (Mello et al. 1993). As SE duration increases, however, BZs are less potent as anticonvulsants (Walton and Treiman 1988; Jones et al. 2002) and neuroprotectants (Pitkänen et al. 2005), and recent data suggest that this could be due to an internalization of $GABA_A$ receptors, as described in the hippocampus, beginning in the first hour of SE (Naylor et al. 2005; Goodkin et al. 2005). The failure of neuroprotection in the hippocampus by diazepam after 3 hours of SE reported previously (Pitkänen et al. 2005) is consistent with our results that diazepam administration after 3 hours of SE did not reduce neurodegeneration in the hippocampus, one week after SE. However, the number of degenerating cells in the amygdala, piriform cortex, and endopiriform nucleus was reduced by diazepam. Thus, the impaired function

and internalization of $GABA_A$ receptors in the hippocampus (Goodkin et al. 2005; Naylor et. al. 2005; Feng et al. 2008) that diminish the efficacy of diazepam may not occur in other limbic structures that retain responsiveness to BZs after prolonged SE.

A reduction of $GABA_A$ receptor expression has not been reported acutely after SE in the amygdala, piriform cortex, or endopiriform nucleus. In fact, Kish et al. (1983) have shown no change in radio-labeled BZ- or GABA-binding 2 hours after SE in the amygdala/piriform cortex area; however, this study also reported no change in the hippocampus. Radio-labeled ligands can bind non-neuronal cell types such as glia; this may account for the difference in the results of Kish et al. (1983) and those by Goodkin et al. (2005), and Naylor et al. (2005), which used electrophysiology and immunohistochemistry to show SE-induced intracellular accumulation/internalization of $GABA_A$ receptors in the hippocampus. Thus, it is unclear at present whether $GABA_A$ receptors are *not* downregulated in the amygdala, piriform cortex, and endopiriform nucleus during prolonged SE, which would account for the neuroprotective effect of diazepam in these brain regions.

A direct neuroprotective effect by diazepam administration after prolonged SE may be difficult to tease apart from its anticonvulsant effect. Because the severity of neuronal loss corresponds to the duration of SE (Lemos and Cavalheiro 1995; Gorter et al. 2003), the termination of SE by diazepam alone may be sufficient to reduce the subsequent neuronal loss without a direct neuroprotective effect of diazepam. The dose of diazepam used in the present study (25 mg/kg) is effective at terminating SE, as determined by disruption of electrographic cortical seizure activity (not shown here). However, the use of deep electrodes would be necessary to determine if subcortical

electrographic seizure activity was also attenuated or terminated with diazepam. Because seizure or spiking activity in subcortical structures can persist even after attenuation of cortical seizure activity (unpublished observations), and, in the hippocampus, $GABA_A$ receptors are internalized within the first hour of SE (Goodkin et al. 2005; Naylor et al. 2005; Feng et al. 2008), the lack of a neuroprotective effect of diazepam in the hippocampus may be explained by a reduced anticonvulsant efficacy of diazepam within this region during prolonged SE.

Pitkänen et al. (2005) reported disease modifying effects of diazepam administered after 3 hours of SE, such as reduced frequency of spontaneous seizures; this was attributed to reduced SE duration compared to that of vehicle-treated rats. Although not analyzed here, we would have expected to find similar results, not only because of SE termination, but also because of the significant reduction of neurodegeneration in all limbic structures studied, apart from the hippocampus.

Because the efficacy of many of the proposed anti-epileptogenic and/or neuroprotective treatments has been analyzed in a limited number of brain areas, we underscore here the need to evaluate structures beyond the hippocampus for neuroprotective efficacy during epileptogenesis. The amygdala, a limbic structure that undergoes extensive neurodegeneration after SE-induced epileptogenesis, responds to benzodiazepine treatment after prolonged SE, when the hippocampus does not. As the mechanisms underlying pharmacoresistance during prolonged SE are beginning to be unraveled in the hippocampus (Goodkin et al. 2005; Naylor et al. 2005; Feng et al. 2008), it is essential to understand the cellular changes that occur in other limbic structures for

assessment of the full potential of anticonvulsant and neuroprotective therapies in animal models of TLE.

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TABLE 1

Quantitative Evaluation (number of FJ+ cells, mm^{-2})				
	KA group	KA+DZP group	*p*	% reduction
Amygdala	105 ± 5	39 ± 11	0.0024	62.7
Hippocampus	18 ± 5	25 ±12	0.640	-
Piriform Cortex	42 ± 10	10 ± 3	0.0107	76.9
Endopiriform Nucleus	194 ± 22	102 ± 28	0.0414	47.33
Qualitative Evaluation Score of FJ Staining				
	KA group	KA+DZP group	*p*	% reduction
Amygdala	2.88 ± 0.50	1.23 ± 0.34	0.0264	57.2
Hippocampus	1.71 ± 0.29	1.50 ±0.46	0.7303	12.2
Piriform Cortex	3.63 ± 0.10	1.30 ± 0.37	0.0009	64.2
Endopiriform Nucleus	3.50 ± 0.40	1.77 ± 0.28	0.0077	49.4

Data from quantitative and qualitative analysis of Fluoro-Jade C staining in different limbic structures from rats 7-9 days after KA-induced SE (KA group) or after KA-induced SE that was terminated 3 hours after its onset, by administration of diazepam (KA +DZP group). Values are presented as mean ± standard error of the mean.

Qualitative scale: 0 = no damage, 1 = minimal damage (0–10%), 2 = mild damage (10–25%), 3 = moderate damage (25–45%), and 4 = severe damage (>45%).

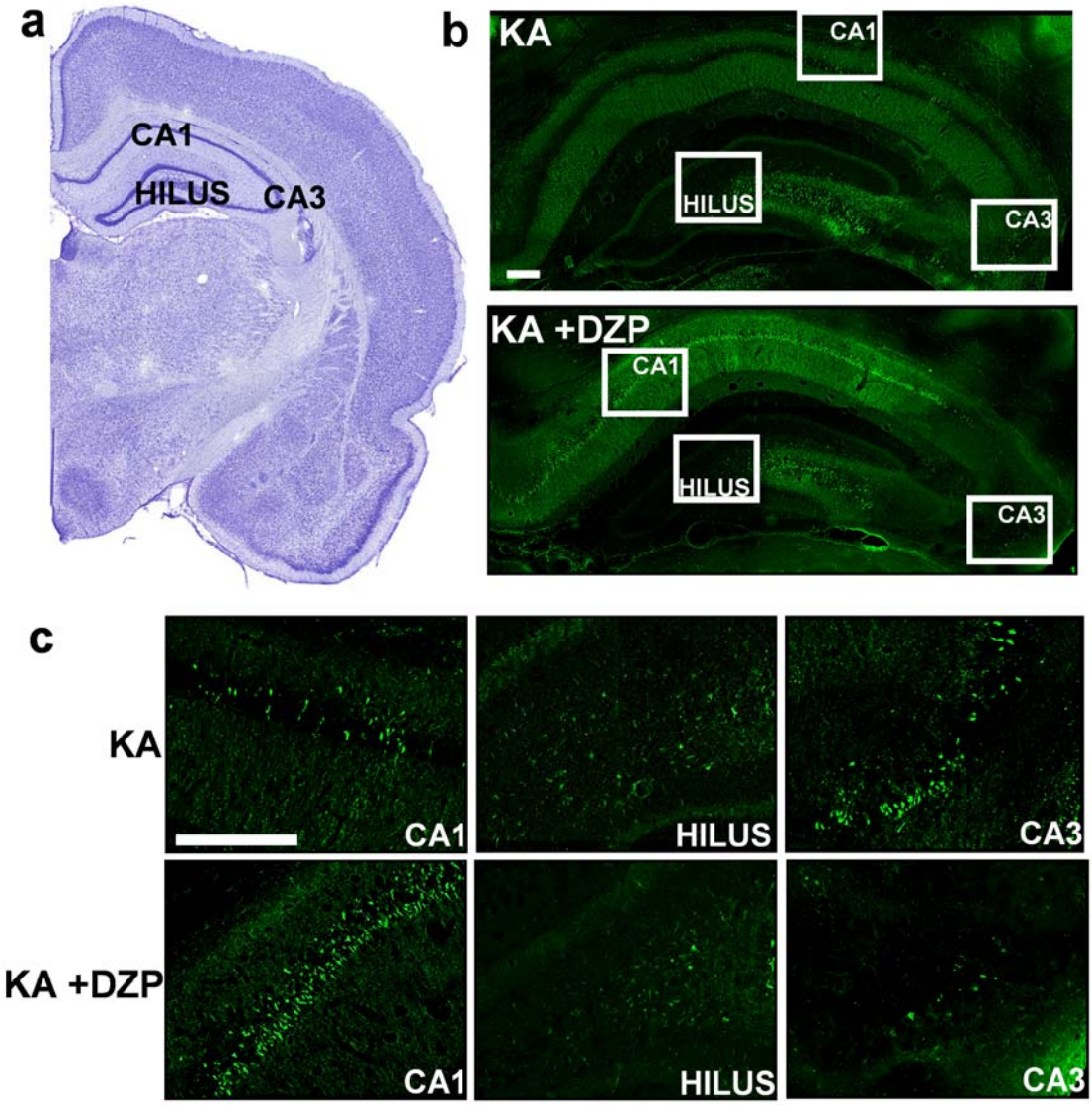


Figure 1. Termination of KA-induced SE by diazepam, 3 hours after its onset, does not reduce neuronal degeneration in the hippocampus during epileptogenesis. Nissl-stained (**a**) and Fluoro-Jade C-stained (**b**) sections demonstrating hippocampal subfields. The CA1, CA3, and hilus regions are shown at higher magnification in (**c**). There was no significant difference in the number of the irreversibly degenerating cells between the group that was administered diazepam (KA +DZP group) 3 hours after the onset of SE,

and the group that was not treated with diazepam (KA group). Staining in this example was performed 7 days after SE. Scale bar = 300 μm.

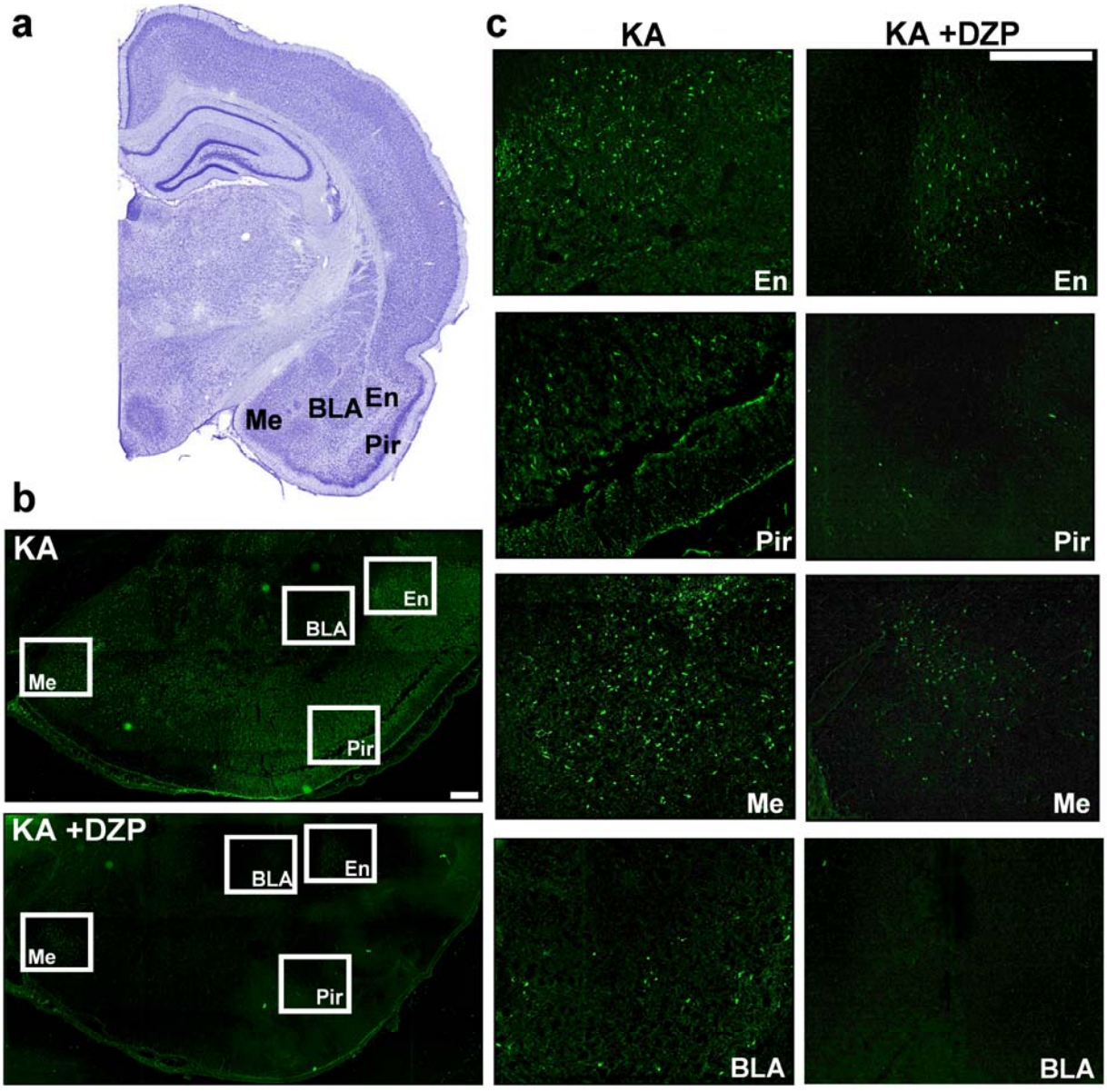


Figure 2. Termination of KA-induced SE by diazepam, 3 hours after its onset, reduces neurodegeneration in the amygdala, piriform cortex, and endopiriform nucleus during epileptogenesis. Nissl-stained (a) and Fluoro-Jade C-stained (b) sections demonstrating the medial (Me), basolateral (BLA) nuclei of the amygdala, the piriform cortex (Pir), and endopiriform nucleus (En). The Fluoro-Jade C-stained sections are

shown at higher magnification in (c). The number of irreversibly degenerating cells in the rats that were administered diazepam (KA +DZP group), 3 hours after the onset of SE, was significantly lower than the number of degenerating cells in the group that was not treated with diazepam (KA group). Staining in this example was performed 7 days after SE. Scale bar = 300 μm.

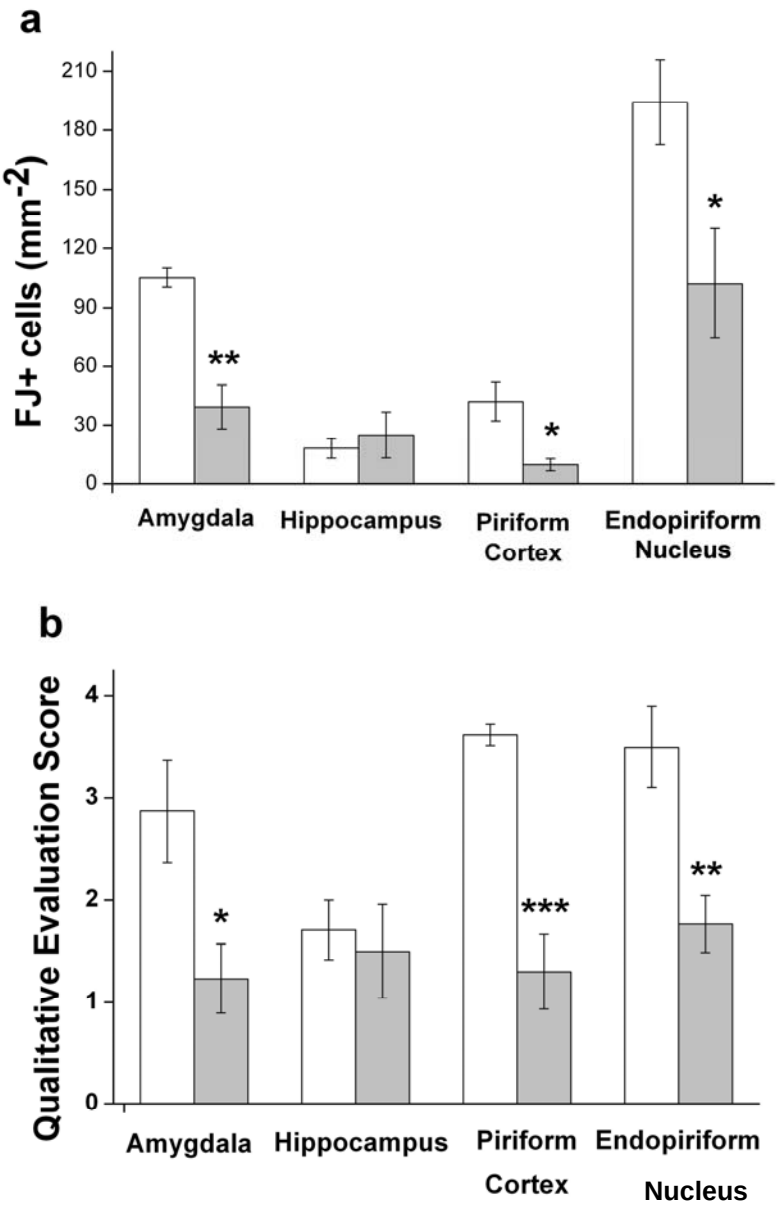


Figure 3. Quantitative and qualitative analysis of Fluoro-Jade C staining reveal that termination of KA-induced SE by diazepam, 3 hours after its onset, reduces neuronal degeneration during epileptogenesis in the amygdala, piriform cortex, and endopiriform nucleus, but not in the hippocampus. a) Comparison of the number of Fluoro Jade-C positive stained cells per mm^2 in the amygdala, hippocampus, piriform cortex

and endopiriform nucleus of rats in which SE induced by kainic acid was not terminated (KA group; n = 4 rats) and rats in which SE was terminated after 3 hours with diazepam (KA +DZP group; n = 5 rats). **b)** Qualitative assessment of the extent of neurodegeneration produced similar relationships between the KA group and the KA +DZP group, as the quantitative evaluation; scale: 0 = no damage; 1 = minimal damage (0-10%); 2 = mild damage (10-25%); 3 = moderate damage (25-45%); 4 = severe damage (>45%). Values are mean ± SEM, * $p<0.05$, ** $p<0.005$; *** $p<0.001$.

CHAPTER 4

Discussion & Future Directions

In patients that develop TLE after an acute brain insult, such as traumatic brain injury or status epilepticus, a seizure-free, epileptogenic latent period is occurs, ranging from days to years after the insult, before the appearance of spontaneous seizures and epilepsy. It is believed that the progressive structural and functional neuronal changes observed during this latent period are the pathophysiological substrate for enhanced seizure susceptibility, and that interruption of these changes will interfere with the development of spontaneous seizures. Consequently, the aim of understanding the mechanisms of epileptogenesis has been at the forefront of epilepsy research during the last decade (Jacobs et al., 2001; Baulac and Pitkänen, 2008).

Using animal models of temporal lobe epilepsy, a large amount of data has been collected, regarding pathological alterations during epileptogenesis, most of which have focused on the hippocampus. Considering the dense intra- and inter-nuclear network of connections within the amygdala, activation of a small portion of the amygdala in pathological conditions may cause excitatory activity to become distributed in parallel among the nuclei of the amygda. Because the amygdala has extensive monosynaptic connections to temporal as well as extra-temporal regions (Amaral et al., 1992), this excitatory activity may then be rapidly propagated to other brain regions, recruiting other structures to form an epileptic circuit. Thus, it is not surprising that the amygdala plays a pivotal role in the spread of seizure activity (White and Price, 1993a,b; Mohapel et al.,

1996), yet changes that take place in the amygdala during epileptogenesis are largely unknown.

The goal of this research was to study alterations in the amygdala that may contribute to the development of spontaneous seizures, as well as to determine the effect of terminating prolonged SE with diazepam on the extent of neurodegeneration during epileptogenesis. We found the inhibitory tone in the BLA is reduced at 7-10 days after KA-SE and before the occurrence of spontaneous seizures in most rats, and the BLA endures significant neuronal loss, of which GABAergic neurons are particularly vulnerable. We also found that SE termination after 3 hours by administration of diazepam results in significant protection from neurodegeneration 7-9 days after KA-SE in the amygdala, endopiriform nucleus, and piriform cortex, but not in the hippocampus. These results emphasize the importance of studying limbic structures in addition to the hippocampus when identifying the neuroprotective potential of therapeutic agents after status epilepticus.

Reduced inhibitory tone and neuronal loss in the BLA after SE-induced epileptogenesis

Hyperexcitable, epileptic circuits arise from an imbalance between excitation and inhibition. Impairments in GABAergic inhibitory transmission have been reported in the BLA of the epileptic amygdala (Gean et al., 1989; Rainnie et al., 1992; Smith and Dudek, 1997; Mangan et al., 2000; for a review see Aroniadou-Anderjaska et al., 2008). Spontaneous IPSPs are reduced or absent in the BLA several months after SE induced by hippocampal stimulation (Mangan et al., 2000). In the BLA of rats several months after

KA-SE, hyperexcitable field responses are observed, and are at least partially due to reduced inhibition (Smith and Dudek, 1997). Several months after the induction of kindled seizures in the amygdala, spontaneous IPSPs are also reduced (Gean et al., 1989; Rainnie et al., 1992). While it is clear that inhibitory transmission is impaired in the BLA of epileptic rats, we report here that the GABAergic system is diminished during epileptogenesis. In the BLA, we found reduced inhibitory tone 7-10 days after SE in KA-SE rats compared to sham rats, demonstrated by a reduction of the frequency and amplitude of sIPSCs recorded in principal neurons. These findings, in addition to decreased frequency of mIPSCs, suggest a reduced inhibitory input to principal cells. The lack of a change in mIPSC amplitude disputes any alterations in the number/density of postsynaptic $GABA_A$ receptors. Thus, during epileptogenesis, the inhibitory transmission of the BLA is impaired, which could be due to loss of interneurons, a lowered probability of transmitter release, and/or a reduced excitatory drive onto the remaining interneurons, but not to a change in the responsiveness of $GABA_A$ receptors.

Neuronal loss has been demonstrated in the amygdala in human patients with TLE (Cendes et al., 1993; Hudson et al., 1993; Wolf et al., 1997; Guerreiro et al., 1999) and in the BLA in rats after KA-SE (Tuunanen et al., 1996, 1999; Pitkänen et al., 1998; Covolan and Mello, 2000). Specifically, a loss of GABAergic neurons has been shown in the BLA two weeks after KA-SE (Tuunanen et al., 1996) and after amygdala kindling (Callahan et al., 1991). We used design-based stereology to quantify the number of total neurons, and the number of GABAergic neurons in the BLA. Stereological estimations offer the benefit of efficiently providing more accurate and reliable results compared to the standard quantitative methodology (West, 2002; Schmitz and Hof, 2005). Notably,

this is the first reported stereological estimation of total neuron number and total GABAergic neuron number of the BLA, in normal physiological conditions as well as during pathophysiological condition of epileptogenesis. Similar to previous studies using non-stereological estimations (McDonald, 1992), we found that GABAergic neurons compromise 14% of the population of total neurons in the BLA. At 7-10 days after KA-SE, we found a loss of 15% of total neurons in the BLA, and an even greater significant loss (43%) of GABAergic neurons in the BLA at this time point in epileptogenesis.

The reduced inhibitory tone in the BLA during epileptogenesis, as observed by recording from BLA pyramidal cells, may be directly associated with the greater loss of GABAergic neurons after KA-SE. It is unlikely that dysfunction of GABAergic neurons contributes to the reduced inhibitory tone, as IPSPs from direct stimulation of nearby neurons are unaltered (Gean et al., 1989; Rainnie et al., 1992). However, future studies should determine if the reduced inhibitory tone is also partially due to reduced excitatory input onto interneurons. Recording excitatory post-synaptic currents in BLA interneurons at this time point in epileptogenesis will accomplish this.

Similar to the susceptibility of GABAergic neurons to cell death after SE, interneurons in the hippocampus are also vulnerable to SE-induced damage (Sloviter, 1987; Best et al., 1993; Obenhaus et al., 1993; Morin et al., 1998; Sun et al., 2007). Why GABAergic neurons are specifically vulnerable to SE-induced injury is not known. Further, some subpopulations of interneurons in the hippocampus are more vulnerable than others (Sloviter, 1987; Best et al., 1993). An impaired ability to buffer glutamate-mediated calcium toxicity during seizures (Sloviter, 1991), and a failure of neuroprotective signaling pathways have been associated with susceptibility of

interneurons to SE-induced cell death in the hippocampus (Choi et al., 2007). Similar mechanisms may also be associated with the vulnerability of subpopulations of GABAergic interneurons in the BLA (Tuunanen et al., 1996). Stereological quantification of sub-types of interneurons in the BLA at this point in epileptogenesis will determine if specific populations of GABAergic neurons are more vulnerable than others to SE-induced injury.

Kainate receptors containing the GluR5 subunit are highly expressed in the BLA compared to other brain regions (Bettler et al., 1990; Li et al., 2001; Braga et al., 2003). We have previously shown that GluR5KRs modulate neuronal excitability in the BLA, such that postsynaptic GluR5KRs on somatodendritic sites of BLA interneurons enhance the probability of GABA release by depolarizing interneurons, and presynaptic GluR5KRs on GABAergic terminals facilitate GABA release when activated by very low concentrations of an agonist, but inhibit GABA release by stronger stimulation ($\geq$ 1 uM) of the GluR5 agonist ATPA (Braga et al., 2003). In the BLA 7-10 days after KA-SE, these effects of GluR5KR activation are significantly weaker than sham rats. While we found reduced expression of the GluR5 subunit, this reduction, and the weakened function of GluR5KR activation may be attributed to loss of interneurons that express the GluR5KR. Although GluR5KR antagonists have anticonvulsant properties in acute seizure models (Smolders et al., 2002; Rogawski et al., 2003), this effect is not expected to be observed during epileptogenesis, when GluR5KR expression is reduced.

Potential compensatory responses in components of the GABAergic system

In situ hybridization, Western blot, and receptor binding studies have shown that SE-induced epileptogenesis involves alterations in the expression of $GABA_A$ receptor subunits in the hippocampus (Gibbs et al., 1997; Schwarzer et al., 1997; Tsunashima et al., 1997; Brooks Kayal et al., 1998; Fritschy et al., 1999; Gilby et al., 2005; Raol et al., 2006) and the amygdala (Gilby et al., 2005; Rocha et al., 2007). We found an elevation in the expression level of the α1 subunit of the $GABA_A$ receptor in the BLA 7-10 days after KA-SE. However, the whole-cell electrophysiology experiments of the present study found no change in the number/density of postsynaptic $GABA_A$ receptors in the BLA after KA-SE, demonstrated by no change in the amplitude of $GABA_A$ mediated mIPSCs. The up-regulation of the $GABA_A$ α1 may lead to more $GABA_A$ receptors containing the α1 subunit expressed at the membrane without affecting the total number/density of $GABA_A$ receptors. Previous studies, however, have reported that benzodiazepine binding in the BLA in control rats was not significantly different from rats one week or 30 days after pilocarpine-induced SE (Rocha et al., 2007) or 40 days after KA-SE (Rocha and Ondarza-Rovira, 1999), suggesting that the up-regulation of $GABA_A$ α1 does not result in more functional synaptic $GABA_A$ receptors containing the α1 subunit at the membrane. The upregulation of $GABA_A$ α1 may be a compensatory response to reduced inhibition, however a failure of receptor assembly, transport, or subsequent expression on the membrane, and/or receptor phosphorylation (Pumain and Laschet, 2006) may occur. The anticonvulsant effects of benzodiazepines are mediated by $GABA_A$ receptors containing the α subunit (Pritchett et al., 1989; Rudolph et al., 1999; Crestani et al., 2000; Da Settimo et al., 2007). Thus, a failure of expression of $GABA_A$

receptors containing the α1 subunit at the membrane may be important mechanism of pharmacoresistance seen in TLE patients (Semah et al., 1998).

Diazepam administration after prolonged SE reduces neurodegeneration in the amygdala, but not in the hippocampus

Atrophy of limbic structures is often reported in TLE patients (Babb and Brown, 1989; Babb 1991; Cendes et al., 1993; Hudson et al., 1993; Williamson et al., 1993; Cendes et al., 1994), as well as in animals after an epileptogenic insult, in models of TLE (Schwob et al,.1980; Tuunanen et al., 1996, 1999; Covolan and Mello, 2000; Chen and Buckmaster, 2005). Unlike the silver method for observing cell damage, we used a marker specific for *irreversibly* degenerating cells (Schmued et al., 1997), to show that the extent of ongoing neurodegeneration during epileptogenesis is greater in the amygdala compared to the hippocampus and piriform cortex. We also show that qualitative assessment of neurodegeneration corresponds to the quantitative analysis of the number of degenerating cells, supporting the reliability of the qualitative scoring method to assess neurodegeneration.

Neurodegeneration of limbic structures after SE is thought to be initiated by excessive glutamate release during prolonged seizure activity, followed by glutamate receptor mediated excitotoxic calcium-influx, triggering cell death signaling pathways. How significant is cell death in the amygdala during epileptogenesis? A strong excitatory network is required to support epileptic circuits. Because extensive neurodegeneration occurs in the amygdala, remaining neurons may lose the ability to produce such a strong excitatory drive necessary for the generation or spread of seizure

activity. However, we have shown that GABAergic interneurons in the BLA are especially vulnerable to SE-induced damage (Qashu et al., 2008). In the BLA, the amygdala nucleus most important in the initiation and propagation of seizure activity in animal models of TLE (White and Price 1993a, b; Mohapel et al., 1996), the percentage of GABAergic neurons of the total neurons is significantly reduced at 7-10 days after SE, indicating that GABAergic neurons are more vulnerable to SE-induced injury than principal cells (Qashu et al., 2008). Although significant cell loss occurs in the amygdala after KA-SE, the ongoing neurodegeneration may shift the excitatory-inhibitory balance during epileptogenesis in favor of hyperexcitability (Smith and Dudek, 1997; Mangan et al., 2000), thereby increasing the predisposition for epileptiform activity.

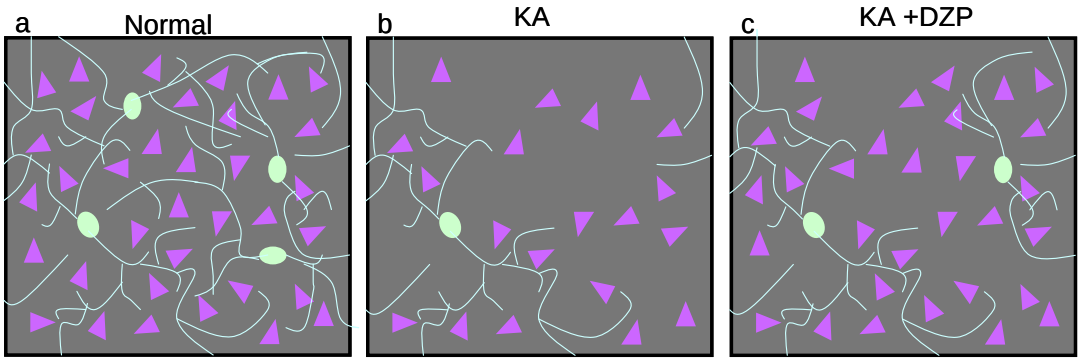
We observed more extensive degeneration occurring in amygdala compared to the hippocampus at this time point in epileptogenesis. Maximal neuronal damage may occur in the hippocampus within a few days after SE, thereafter tapering off (Schmued et al., 1997). Thus, the total amount of neuronal loss relative to other limbic structures, which was not reported here, may give contradictory results.

The finding that intervention of prolonged SE with the benzodiazepine, diazepam, significantly reduces neurodegeneration in the amygdala and other limbic structures associated with epileptogenesis provides useful information for neuroprotective strategies after SE and during epileptogenesis. Benzodiazepine sensitive receptors are densely located in limbic structures (Niehoff and Kuhar, 1983; Fritschy and Möhler, 1995), are the first line treatment for termination of status epilepticus in humans (Chen and Wasterlain, 2006), and to reduce mortality after prolonged SE in animal models of TLE (Mello et al., 1993). As SE duration increases, however, benzodiazepines are less potent

as anticonvulsants (Jones et al., 2002) and neuroprotectants (Pitkänen, 2005), and recent data suggests that this is due to an internalization of $GABA_A$ receptors, as described in the hippocampus, beginning in the first hour of SE (Goodkin et al., 2005; Naylor et al., 2005). The failure of neuroprotection in the hippocampus by diazepam after 3 hours of SE reported previously (Pitkänen 2005) coincides with our results that diazepam administration after 3 hours of SE did not reduce neurodegeneration one week after SE in the hippocampus. However, the number of degenerating cells in the amygdala, piriform cortex, and endopiriform nucleus was reduced when diazepam was used even 3 hours after SE compared to SE that was not terminated with diazepam (Figure 1). Thus, the plastic mechanisms that occur in the hippocampus that diminish the efficacy of diazepam may not occur in other limbic structures that retain responsiveness after prolonged SE. Likewise, a reduction of $GABA_A$ receptor expression has not been reported acutely after SE in the amygdala, piriform cortex, or endopiriform nucleus. Future studies should investigate whether $GABA_A$ receptors are internalized within the first hour of SE in the amygdala. A reduction of mIPSC amplitude will suggest a reduction of functional membrane $GABA_A$ receptors. Western blot analysis of surface expression compared to total expression of $GABA_A$ receptor subunits to determine specifically if benzodiazepine-sensitive $GABA_A$ receptors are internalized.

The neuroprotective effect by diazepam administration after prolonged SE may be due to SE termination, rather than to a direct anticonvulsant effect by diazepam. The severity of neuronal loss corresponds to the duration of SE (Lemos and Cavalheiro, 1995; Gorter et al., 2003), thus, termination of SE alone may be sufficient to reduce the subsequent neuronal loss. After three hours of SE, the dose of diazepam used in the

present study (25 mg/kg) is effective at terminating cortical seizure activity within one hour of administration. To be sure that subcortical electrographic seizure activity was



also attenuated with this dose of diazepam, future studies should employ deep brain electrodes in the EEG recordings.

Figure 1. GABAergic interneurons in the BLA are selectively vulnerable to damage after KA-SE, compared to pyramidal cells. (a) In the normal BLA, interneurons (blue cells) contribute 15% to the total number of neurons, together with pyramidal cells (purple cells). For simplification, pyramidal cell projections are not shown. (b) After SE induced by kainic acid, the BLA undergoes extensive neurodegeneration, to include a 20-50% loss of total neurons but a 75% loss of interneurons (Tuunanen, et al., 1996). (c) Diazepam administration to terminate status epiletpicus after 3 hours is neuroprotective in the BLA, such that there is only a 15% loss of total neurons, but a significant loss of GABAergic interneurons (43%) still occurs.

Implications for epileptogenesis

The present study reports evidence of one mechanism, reduced inhibitory activity in the BLA, which may shift the balance between excitatory and inhibitory transmission during epileptogenesis, thereby promoting the formation of a hyperexcitable, epileptic circuit. We found that GABAergic neurons in the BLA are particularly susceptible to SE-induced injury, and the subsequent reduction of inhibitory input in the BLA is insufficiently compensated by mechanisms to counterbalance the loss. The greater vulnerability and subsequent neurodegeneration of GABAergic neurons after KA-SE may confer to an imbalance in the excitability of the BLA, and play an important role in the mechanisms of epileptogenesis. Indeed, after amygdala kindling, when epileptogenic changes are thought to occur, stimulation of the BLA more readily generates SE compared to other limbic structures (Mohapel et al., 1996). During epileptogenesis, loss of interneurons may lower the threshold for seizure generation in the BLA, and, because the BLA has a more extensive projection system than other amygdala nuclei (Krettek and Price, 1978, Amaral et al., 1992), including the most efferents to the hippocampus (Pitkänen, 2000), the hyperexcitable BLA has the potential to recruit and involve other structures of the limbic circuit for the spread of seizure activity. Thus, net failure of inhibitory transmission due to loss of GABAergic neurons in the BLA may play an important role in epileptogenesis, and the development of epilepsy.

Studies that have shown success in reducing the effects of epileptogenesis have largely failed to prevent epileptogenesis (André et al., 2001; Brandt et al., 2003; Narkilahti et al., 2003; Pitkänen, et al., 2005). Pitkänen, et al. (2005) reported disease modifying effects of SE termination after 3 hours with diazepam, such that rats in which

SE was terminated at 3 hours displayed a reduced frequency of spontaneous recurrent seizures compared to vehicle treated rats in which SE was not terminated, even though the extent of hippocampal neurodegeneration between the two groups was not different. We would expect similar results from animals in our study, not only because of SE termination, but also because of the significant reduction of neurodegeneration in limbic structures that are important for epileptogenesis. Because the efficacy of many of the proposed anti-epileptogenic and/or neuroprotective treatments have been analyzed in a limited number of brain areas, we underscore here the need to evaluate structures beyond the hippocampus for neuroprotective efficacy. The amygdala, a limbic structure that undergoes extensive neurodegeneration after SE-induced epileptogenesis, responds to benzodiazepine treatment after prolonged SE, when the hippocampus does not. Thus, the amygdala may be a more promising target for neuroprotective therapies during epileptogenesis.

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Appendix

Design-based stereology

The underlying objective of using stereological methods is to obtain an unbiased estimation of geometric properties of three-dimensional objects, such as area, volume, length, and population size from two-dimensional planar sections (Gundersen and Jensen, 1987; West, 2002). The sampling scheme in stereology is "designed", or defined *a priori*, and is independent of the size, shape, spatial orientation, or spatial distribution of the object of interest, to ensure that all objects have the same probability of being counted, and are counted once and only once. Previous model-based quantification analyses relied on assumptions of geometric features, and used formulas to correct for truncation or over-estimation (Abercrombie and Johnson, 1946; Weibel and Gomez, 1962). Incorrect assumptions or misuse of formulas are potential sources of error using model-based methods. Excluding assumptions of geometric properties of the objects to be counted in stereology reduces the potential for errors created when using model-based methods, providing results that are more accurate and reliable.

To use stereology as a method of estimating geometric properties of an object of interest, the entire anatomical region containing the object must be available and distinctly delineated based on natural boundaries. "Systematic, random sampling" in stereology refers to the random selection of a series of systematically cut sections containing the object of interest, for example randomly selecting the third series of section from a set of five series, and the random selection of systematic counting sites in any particular section. Counting the object of interest in a subset of the entire structure

with systematic random sampling permits efficient quantification, while maintaining unbiased estimations.

The past few decades have seen a marked increase in the use of stereology in the biological sciences to understand the morphology of organs or structures, particularly in the central nervous system (Gundersen et al., 1999; Glaser and Glaser, 2000; West, 2002). Examples of the use of stereology in neuroscience include determining the length of axons, the volume of brain regions, or, most commonly, the estimation of total population of cells in a particular brain structure. In the following paragraphs, a detailed illustration is presented of the quantification of the total number of neurons in a brain structure using the Optical Fractionator probe of the StereoInvestigator software (Williston, VT), for counting objects in a subfraction of the total volume of the structure.

Quantification of the total number of neurons in the rat basolateral amygdala (BLA)

Thick coronal sections (40 μm) containing the amygdala are cut from fixed, frozen tissue dissected from a young adult rat brain. Because the BLA is a relatively small structure, in order to have enough sections for a confident stereological estimation (ideally ≥10), sections are cut in a series of six, to increase the number of sections in the series that contains the amygdala. Alternatively, for a relatively large structure, such as the hippocampus, there are more rostro-caudal sections that contain the hippocampus; therefore a series of ten may be used. A random series of the six series is selected for mounting on slides and cresyl violet staining for Nissl to identify neurons (Figure 1a).

Because cresyl violet staining involves incubating the slides in a series of alcohol solutions for dehydration, extensive shrinkage of the tissue occurs. The expected amount of shrinkage from cresyl violet staining is 30-50%. For example, tissue sections cut at 40 μm might be only 20-28 μm thick after staining. In preparation of sections for stereology, it is vital to minimize the extent of tissue shrinkage, to maximize the amount of tissue available for analysis in the z-axis, called the disector height (ideally the disector height is ≥20 μm for counting neurons).

Using a low-magnification objective (2.5× objective), the BLA is traced from each section containing the structure, from mounted Nissl-stained sections (Figure 1). Sampling parameters, such as the grid size, counting frame, and disector height for each counting site are defined before counting, and should remain the same for all sections for each animal. Two hundred is the minimum number of objects to count for each animal; higher numbers can be counted to reduce systematic errors, but is more time-consuming. Thus, several pilot studies must be done in order to determine the most efficient sampling parameters, while maintaining confidence in the total estimation by reducing the error contributed by methodological variance.

The counting frame for each counting site (Figure 2) is dependent upon the relative size of the object of interest (a neuron). It is ideal to be able to count 1-5 objects for each grid size. Based on the size of the neurons in the BLA, the counting frame for the sections is 35 μm × 35 μm. The counting grid (Figure 3) determines the space between the two counting sites, such that a larger grid size will increase the space between counting sites, and reduce the number of counting sites, and a smaller grid size will decrease the space between counting sites, thereby increasing the number of

counting sites. For counting the total number of neurons in the BLA, the counting grid size is 190 μm × 190 μm. The disector height delineates the depth of tissue along the Z-axis in which cells are selected, and depends on the thickness of the tissue, and the size of the guard zones (Figure 4). Cutting thick sections and minimizing the extent of tissue shrinkage allows for a larger disector height. Due to extensive shrinkage of Nissl-stained sections, the disector height for counting neurons in the BLA is 12 μm. Guard zones are at the top and bottom of the disector, and serve the purpose of avoiding counting areas of the section where cells may have been plucked out during sectioning. The height of the guard zone is ideally set at half the diameter of the object of interest, but should be at least 2 μm.

Once the parameters are set, counting begins at the first counting site on the counting frame of the first section (Figure 2). At a higher magnification (63× or 100× oil objectives), the top and bottom of the section are determined and measured, by focusing through the optical planes with the z-focus. Beginning at the top of the section, the z-focus is used to pass through the guard zone and into the disector height (Figure 4). Cells are counted if a unique point of the object of interest (defined before counting) passes through the disector height while moving through the optical planes. For counting neurons from Nissl-stained sections in the BLA, the unique point is the top of the cell. Alternatively, the nucleus of the cell can be used as the unique point. Furthermore, to be counted, cells must lie completely within the counting frame, or cross an inclusion line, but are not counted if they lie completely outside the counting frame, or if they intersect an exclusion line. The inclusion and exclusion lines are essential in eliminating the bias of selecting cells of a certain shape, and ensuring that cells are counted once and only

once. After all satisfactory cells have been selected for a counting site, an automatic stage on the microscope moves automatically to the next counting site.

Once all sites in all counting sites from all sections have been counted (Figure 5), the population estimate, N is calculated as the reciprocal of the volume fraction multiplied by the sum of the total objects counted (ΣQ) from all counting sites, from all sections containing the BLA. The volume fraction, or the volume of the entire structure that was actually analyzed, is calculated based on the sectioning and sampling parameters:

Volume fraction = (ssf)(asf)(hsf)

The volume fraction is calculated as the product of the section interval (ssf), the area sampling fraction (asf), and the height sampling fraction (hsf). For calculating the total number of neurons in the amygdala, in the example above, the section interval is 6. The area sampling fraction, or asf, is calculated by the grid size (e.g. 35 μm) divided by the counting frame area (e.g. 190 μm × 190 μm). Finally, the height sampling fraction is the disector height (12 μm) is divided by the average section thickness (measured at each counting site while counting).

Thus, the total population estimate (N) is: $\mathbf{N = (1/\mathit{ssf}) \times (1/\mathit{asf}) \times (1/\mathit{hsf}) \times \Sigma Q}$

Summary

Stereological assessments are accurate and efficient means of quantifying geometric properties of three-dimensional, irregularly-shaped objects from two-dimensional planar sections. Without relying on object size, shape, orientation, or distribution, pre-determined sectioning and sampling parameters and strict counting rules

maintain unbiased counting to provide reliable estimations of total populations. These methods have been, and continue to be employed in biological research, including neuroscience, to contribute to the understanding of functional and morphological features of organ structure and pathology.

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Figures

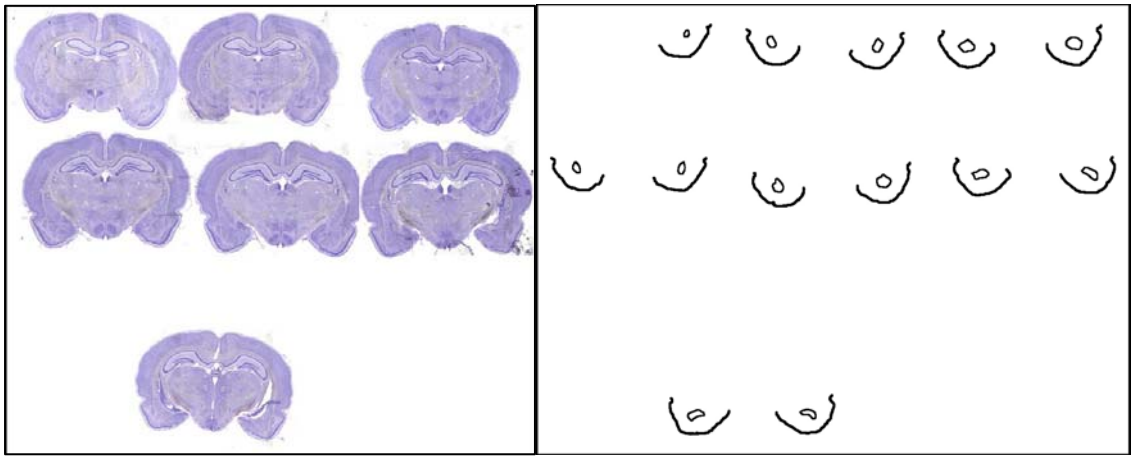

Figure 1. Tracings of the BLA from Nissl-stained sections. For stereological quantification of total number of neurons in the BLA, using thick sections **(a)**, cut at 40 um, tracings **(b)** of each BLA in each section derived from a series of sections are made using a low magnification objective.

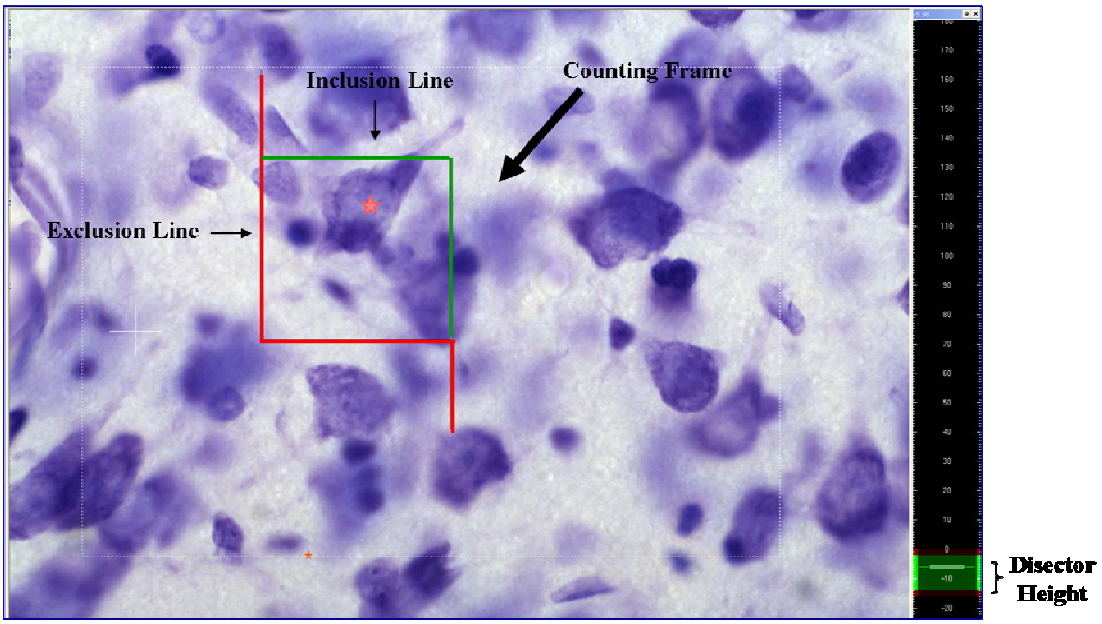

Figure 2. Counting site for stereological quantification of Nissl-stained neurons in the BLA. The counting frame is 35 µm × 35 µm, and is comprised of two green inclusion lines and two red exclusion lines to determine whether or not neurons are counted. Pictured is a neuron (starred) that is counted because its unique point (the top of the cell) comes into focus within the optical plane in the disector (not shown), and crosses a green inclusion line, but does not cross a red exclusion line.

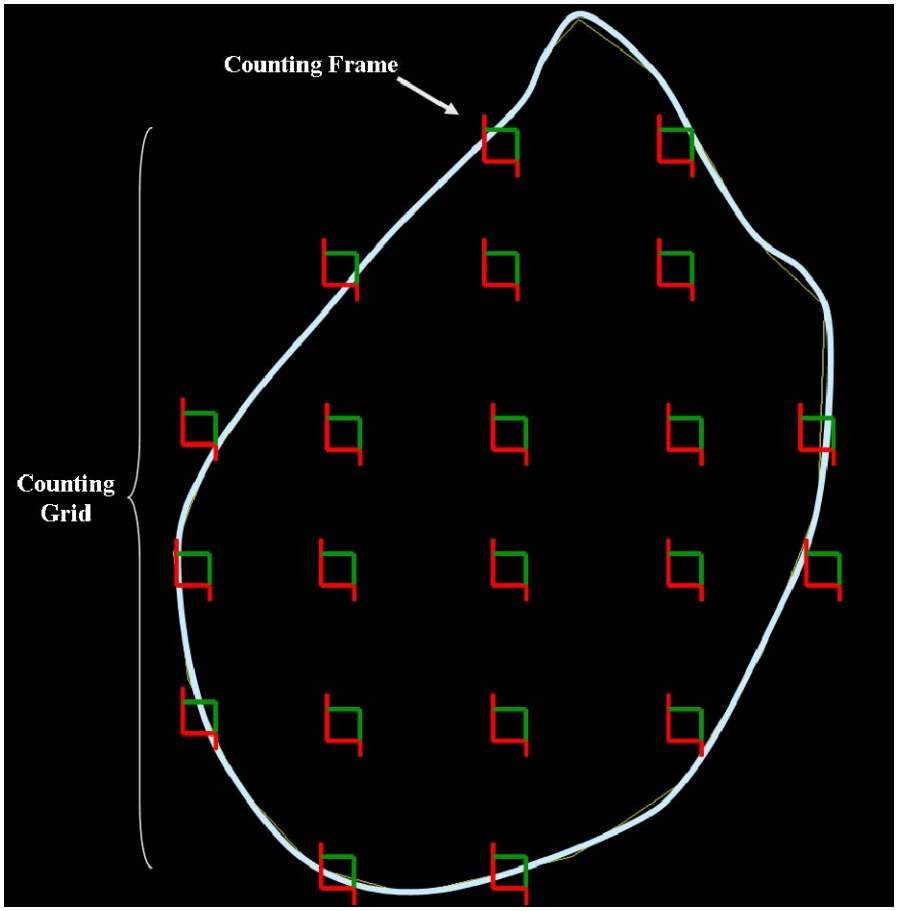

Figure 3. Counting grid superimposed over tracing of BLA. Counting frames (35 µm × 35 µm) are systematically spaced 190 µm × 190 µm apart along a counting grid. The counting grid is randomly superimposed over the tracing of the BLA.

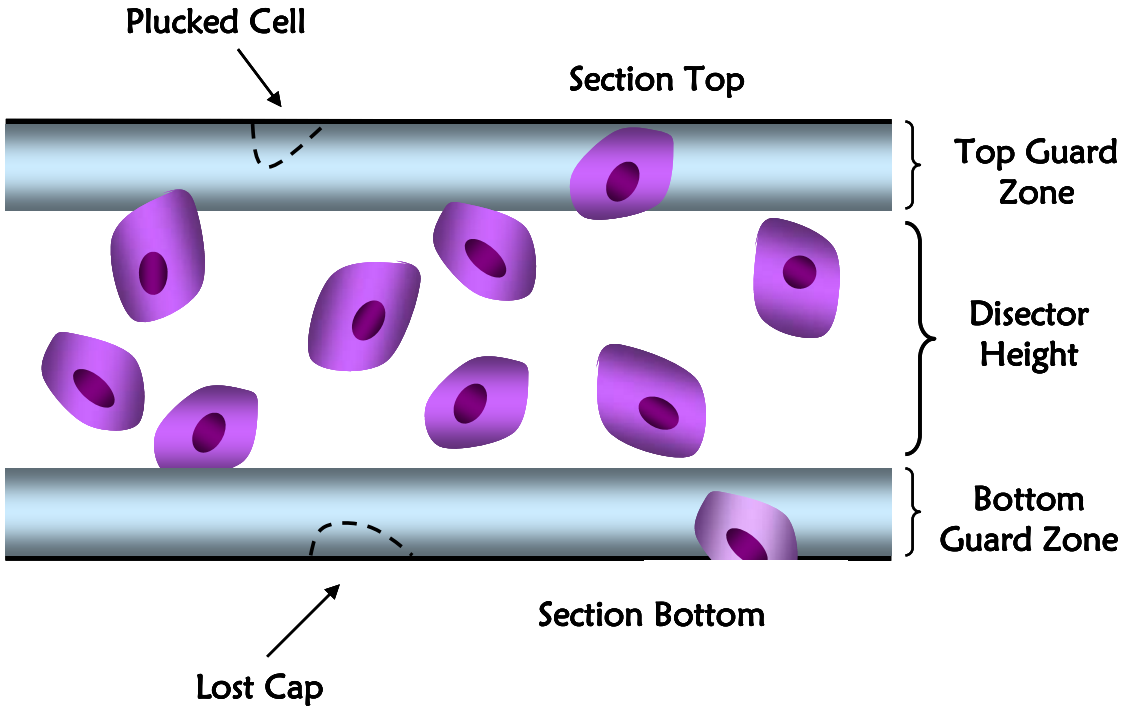

Figure 4. Disector height and guard zones in Nissl-stained sections. Transverse view of Nissl-stained sections demonstrating that the disector height (12 µm), where cells are counted, is bounded by guard zones (2 µm) that serve to avoid counting the area of a section where cells may be removed during sectioning, from the top (plucked cell) or the bottom (lost cap).

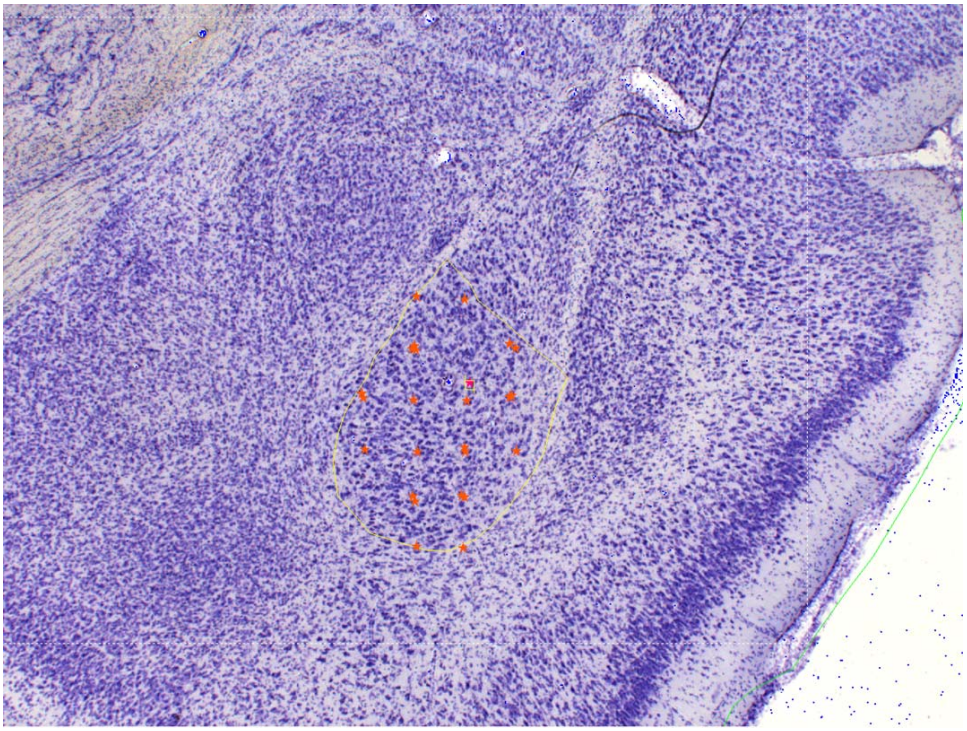

Figure 5. Nissl-stained section of a BLA in which stereological counts have been completed. Counted neurons (orange dots) can be identified on the counting grid.